

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re the application of:

SCHETTERS Theodorus P.M.

CARCY Bernard P.D.

DRACULOVSKI Pascal R.

GORENFLOT Andre F.

Serial Number: To be assigned Group Art Unit: To be assigned

Filed: concurrently herewith Examiner: To be assigned

For: BABESIA CANIS VACCINE

Corresponding to: EP01200816.5, filed 06 March, 2001

**PRELIMINARY AMENDMENT**

Assistant Commissioner of Patents  
Washington, D.C. 20231

February 27, 2002

Dear Sir,

Applicants are hereby including this preliminary amendment to the enclosed US filing of European patent application number 01200816.5 filed on March 6, 2001. Filed with this preliminary amendment is a copy of the original specification from the European patent application, a certified copy of the priority document filed in the European patent office, declarations and power of attorney from all the inventors, assignments from all the inventors, and a sequence listing. Please charge all required fees to Deposit Account No. 02-2334.

**I. In the Claims (Clean Sheet)**

23. A nucleic acid sequence encoding a 15 kD *Babesia canis* associated protein or an immunogenic fragment of said protein, said protein or immunogenic fragment thereof having at least 80% homology with the amino acid sequence as depicted in SEQ ID NO:2.

24. The nucleic acid sequence of Claim 23 having at least 90% homology with the amino acid sequence as depicted in SEQ ID NO:2.

25. The nucleic acid sequence of Claim 23 having at least 95% homology with the amino acid sequence as depicted in SEQ ID NO:2.

26. A cDNA comprising a nucleic acid sequence according to Claim 23.

27. A recombinant DNA molecule comprising a nucleic acid sequence according to Claim 23, under the control of a functionally linked promoter.

28. A recombinant DNA molecule comprising a cDNA comprising a nucleic acid sequence according to Claim 23, under the control of a functionally linked promoter.

29. A live recombinant carrier comprising a cDNA comprising a nucleic acid sequence according to Claim 23.

30. A live recombinant carrier comprising a recombinant DNA molecule selected from the group consisting of a

nucleic acid sequence according to Claim 23 and a cDNA comprising a nucleic acid sequence according to Claim 23, under the control of a functionally linked promoter.

31. A host cell comprising a sequence selected from the group consisting of a nucleic acid sequence according to Claim 23; a cDNA fragment comprising a nucleic acid sequence according to Claim 23; a recombinant DNA molecule comprising a sequence selected from the group consisting of a nucleic acid sequence according to Claim 23 and a cDNA comprising a nucleic acid sequence according to Claim 23, under the control of a functionally linked promoter; and, a live recombinant carrier comprising a carrier selected from the group consisting of a nucleic acid sequence according to Claim 23 and a recombinant DNA molecule comprising a sequence selected from the group consisting of a nucleic acid sequence according to Claim 23 and a cDNA comprising a nucleic acid sequence according to Claim 23, under the control of a functionally linked promoter.
32. A *Babesia canis* associated protein, said protein having a molecular weight of 15 kD and comprising an amino acid sequence that is at least 80% homologous to the amino acid sequence as depicted in SEQ ID NO:2 or an immunogenic fragment of said protein.
33. The *Babesia canis* associated protein of claim 32 wherein the amino acid sequence is at least 85% homologous to the amino acid sequence as depicted in SEQ ID NO: 2, or an immunogenic fragment of said protein.

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34. The *Babesia canis* associated protein of claim 32 wherein the amino acid sequence is at least 90% homologous to the amino acid sequence as depicted in SEQ ID NO: 2, or an immunogenic fragment of said protein.

35. The *Babesia canis* associated protein of claim 32 wherein the amino acid sequence is at least 95% homologous to the amino acid sequence as depicted in SEQ ID NO: 2, or an immunogenic fragment of said protein.

36. A vaccine for combating *Babesia canis* infections, comprising an immunogen selected from the group consisting of a nucleic acid sequence according to Claim 23; a cDNA comprising a nucleic acid sequence according to Claim 23; a recombinant DNA molecule comprising a sequence selected from the group consisting of a nucleic acid sequence according to Claim 23 and a cDNA comprising a nucleic acid sequence according to Claim 23, under the control of a functionally linked promoter; a live recombinant carrier selected from the group consisting of a nucleic acid sequence according to Claim 23 and a recombinant DNA molecule selected from the group consisting of a cDNA comprising a nucleic acid sequence according to Claim 23 and a nucleic acid sequence according to Claim 23, under the control of a functionally linked promoter; and, a host cell comprising a sequence selected from the group consisting of a nucleic acid sequence according to Claim 23, a cDNA fragment comprising a nucleic acid sequence according to Claim 23, a recombinant DNA molecule comprising a sequence selected from the group consisting of a nucleic acid sequence according to Claim 23 and a cDNA comprising a nucleic acid sequence according to Claim 23,

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under the control of a functionally linked promoter, and, a live recombinant carrier comprising a carrier selected from the group consisting of a nucleic acid sequence according to Claim 23 and a recombinant DNA molecule comprising a sequence selected from the group consisting of a nucleic acid sequence according to Claim 23 and a cDNA comprising a nucleic acid sequence according to Claim 23, under the control of a functionally linked promoter.

37. The vaccine of claim 36 further comprising an adjuvant.
38. The vaccine of claim 36 further comprising an additional antigen derived from a virus or microorganism pathogenic to dogs or a nucleic acid sequence encoding said antigen.
39. The vaccine according to claim 38, wherein said virus or micro-organism pathogenic to dogs is selected from the group of *Ehrlichia canis*, *Babesia gibsoni*, *vogeli*, *rossi*, *Leishmania donovani*-complex, Canine parvovirus, Canine distempervirus, *Leptospira interrogans* serovar *canicola*, *icterohaemorrhagiae*, *pomona*, *grippotyphosa*, *bratislava*, Canine hepatitisvirus, Canine parainfluenzavirus, rabies virus, *Hepatozoon canis* and *Borrelia burgdorferi*.
40. A vaccine for combating *Babesia canis* infections, comprising antibodies against a protein selected from the group consisting of at least one of the proteins of claims 32-35, or immunogenic fragment thereof.

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41. A diagnostic test for the detection of *Babesia canis* associated RNA wherein the test comprises a nucleic acid sequence according to Claim 23, a nucleotide sequence that is complementary to said nucleic acid sequence, and a fragment thereof having a length of at least 12 nucleotides.

42. A diagnostic test for the detection of antibodies against *Babesia canis* associated antigenic material, wherein said test comprises a protein or an immunogenic fragment thereof as defined in claims 32-35.

43. A diagnostic test for the detection of *Babesia canis* associated antigenic material, wherein said test comprises antibodies against a protein or an immunogenic fragment thereof as defined in claims 32-35.

44. A nucleic acid sequence encoding a 32 kD *Babesia canis* associated protein or an immunogenic fragment of said protein, said protein or immunogenic fragment thereof having at least 80% homology with the amino acid sequence as depicted in SEQ ID NO:4.

45. The nucleic acid sequence of Claim 44 having at least 90% homology with the amino acid sequence as depicted in SEQ ID NO:4.

46. The nucleic acid sequence of Claim 44 having at least 95% homology with the amino acid sequence as depicted in SEQ ID NO:4.

47. A cDNA comprising a nucleic acid sequence according to Claim 44.

48. A recombinant DNA molecule comprising a nucleic acid sequence according to Claim 44, under the control of a functionally linked promoter.

49. A recombinant DNA molecule comprising a cDNA comprising a nucleic acid sequence according to Claim 44, under the control of a functionally linked promoter.

50. A live recombinant carrier comprising a cDNA comprising a nucleic acid sequence according to Claim 44.

51. A live recombinant carrier comprising a recombinant DNA molecule selected from the group consisting of a nucleic acid sequence according to Claim 44 and a cDNA comprising a nucleic acid sequence according to Claim 44, under the control of a functionally linked promoter.

52. A host cell comprising sequence selected from the group consisting of a nucleic acid sequence according to Claim 44; a cDNA fragment comprising a nucleic acid sequence according to Claim 44; a recombinant DNA molecule comprising a sequence selected from the group consisting of a nucleic acid sequence according to Claim 44 and a cDNA comprising a nucleic acid sequence according to Claim 44, under the control of a functionally linked promoter; and, a live recombinant carrier comprising a carrier selected from the group consisting of a nucleic acid sequence according to Claim 44 and a recombinant DNA molecule comprising a sequence selected from the group consisting of a nucleic

acid sequence according to Claim 44 and a cDNA comprising a nucleic acid sequence according to Claim 44, under the control of a functionally linked promoter.

53. A *Babesia canis* associated protein, said protein having a molecular weight of 32 kD and comprising an amino acid sequence that is at least 80% homologous to the amino acid sequence as depicted in SEQ ID NO:4 or an immunogenic fragment of said protein.

54. The *Babesia canis* associated protein of claim 53 wherein the amino acid sequence is at least 85% homologous to the amino acid sequence as depicted in SEQ ID NO: 4, or an immunogenic fragment of said protein.

55. The *Babesia canis* associated protein of claim 53 wherein the amino acid sequence is at least 90% homologous to the amino acid sequence as depicted in SEQ ID NO: 4, or an immunogenic fragment of said protein.

56. The *Babesia canis* associated protein of claim 53 wherein the amino acid sequence is at least 95% homologous to the amino acid sequence as depicted in SEQ ID NO: 4, or an immunogenic fragment of said protein.

57. A vaccine for combating *Babesia canis* infections, comprising an immunogen selected from the group consisting of a nucleic acid sequence according to Claim 44; a cDNA fragment comprising a nucleic acid sequence according to Claim 44; a recombinant DNA molecule comprising a sequence selected from the group consisting of a nucleic acid sequence according to Claim 44 and a cDNA comprising a

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nucleic acid according to Claim 44, under the control of a functionally linked promoter; a live recombinant carrier selected from the group consisting of a nucleic acid sequence according to Claim 44 and a recombinant DNA molecule selected from the group consisting of a cDNA comprising a nucleic acid sequence according to Claim 44 and a nucleic acid sequence according to Claim 44, under the control of a functionally linked promoter; and, a host cell comprising sequence selected from the group consisting of a nucleic acid sequence according to Claim 44, a cDNA fragment comprising a nucleic acid sequence according to Claim 44, a recombinant DNA molecule comprising a sequence selected from the group consisting of a nucleic acid sequence according to Claim 44 and a cDNA comprising a nucleic acid sequence according to Claim 44, under the control of a functionally linked promoter, and, a live recombinant carrier comprising a carrier selected from the group consisting of a nucleic acid sequence according to Claim 44 and a recombinant DNA molecule comprising a sequence selected from the group consisting of a nucleic acid sequence according to Claim 44 and a cDNA comprising a nucleic acid sequence according to Claim 44, under the control of a functionally linked promoter.

58. The vaccine of claim 57 further comprising an adjuvant.

59. The vaccine of claim 57 further comprising an additional antigen derived from a virus or microorganism pathogenic to dogs or a nucleic acid sequence encoding said antigen.

60. The vaccine according to claim 59, wherein said virus or micro-organism pathogenic to dogs is selected from the group of *Ehrlichia canis*, *Babesia gibsoni*, *vogeli*, *rossi*, *Leishmania donovani*-complex, Canine parvovirus, Canine distempervirus, *Leptospira interrogans* serovar *canicola*, *icterohaemorrhagiae*, *pomona*, *grippotyphosa*, *bratislava*, Canine hepatitisvirus, Canine parainfluenzavirus, rabies virus, *Hepatozoon canis* and *Borrelia burgdorferi*.

61. A vaccine for combating *Babesia canis* infections, comprising antibodies against a protein selected from the group consisting of at least one of the proteins of Claims 53-56, or immunogenic fragment thereof.

62. A diagnostic test for the detection of antibodies against *Babesia canis* associated antigenic material, wherein said test comprises a protein or an immunogenic fragment thereof as defined in claims 52-55

63. A diagnostic test for the detection of *Babesia canis* associated antigenic material, wherein said test comprises antibodies against a protein or an immunogenic fragment thereof as defined in claims 52-55.

**II. In the Claims (Marked Version)**

Please cancel claims 1-22 without prejudice or disclaimer. Applicants are canceling claims 1-22 to present the identical claims in a proper form for U.S. examination

Please insert the following claims:

- - 23. A nucleic acid sequence encoding a 15 kD *Babesia canis* associated protein or an immunogenic fragment of said protein, said protein or immunogenic fragment thereof having at least 80% homology with the amino acid sequence as depicted in SEQ ID NO:2.

24. The nucleic acid sequence of Claim 23 having at least 90% homology with the amino acid sequence as depicted in SEQ ID NO:2.

25. The nucleic acid sequence of Claim 23 having at least 95% homology with the amino acid sequence as depicted in SEQ ID NO:2.

26. A cDNA comprising a nucleic acid sequence according to Claim 23.

27. A recombinant DNA molecule comprising a nucleic acid sequence according to Claim 23, under the control of a functionally linked promoter.

28. A recombinant DNA molecule comprising a cDNA comprising a nucleic acid sequence according to Claim 23, under the control of a functionally linked promoter.

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29. A live recombinant carrier comprising a cDNA comprising a nucleic acid sequence according to Claim 23.

30. A live recombinant carrier comprising a recombinant DNA molecule selected from the group consisting of a nucleic acid sequence according to Claim 23 and a cDNA comprising a nucleic acid sequence according to Claim 23, under the control of a functionally linked promoter.

31. A host cell comprising a sequence selected from the group consisting of a nucleic acid sequence according to Claim 23; a cDNA fragment comprising a nucleic acid sequence according to Claim 23; a recombinant DNA molecule comprising a sequence selected from the group consisting of a nucleic acid sequence according to Claim 23 and a cDNA comprising a nucleic acid sequence according to Claim 23, under the control of a functionally linked promoter; and, a live recombinant carrier comprising a carrier selected from the group consisting of a nucleic acid sequence according to Claim 23 and a recombinant DNA molecule comprising a sequence selected from the group consisting of a nucleic acid sequence according to Claim 23 and a cDNA comprising a nucleic acid sequence according to Claim 23, under the control of a functionally linked promoter.

32. A *Babesia canis* associated protein, said protein having a molecular weight of 15 kD and comprising an amino acid sequence that is at least 80% homologous to the amino acid sequence as depicted in SEQ ID NO:2 or an immunogenic fragment of said protein.

The United States Patent and Trademark Office

33. The *Babesia canis* associated protein of claim 32 wherein the amino acid sequence is at least 85% homologous to the amino acid sequence as depicted in SEQ ID NO: 2, or an immunogenic fragment of said protein.

34. The *Babesia canis* associated protein of claim 32 wherein the amino acid sequence is at least 90% homologous to the amino acid sequence as depicted in SEQ ID NO: 2, or an immunogenic fragment of said protein.

35. The *Babesia canis* associated protein of claim 32 wherein the amino acid sequence is at least 95% homologous to the amino acid sequence as depicted in SEQ ID NO: 2, or an immunogenic fragment of said protein.

36. A vaccine for combating *Babesia canis* infections, comprising an immunogen selected from the group consisting of a nucleic acid sequence according to Claim 23; a cDNA comprising a nucleic acid sequence according to Claim 23; a recombinant DNA molecule comprising a sequence selected from the group consisting of a nucleic acid sequence according to Claim 23 and a cDNA comprising a nucleic acid sequence according to Claim 23, under the control of a functionally linked promoter; a live recombinant carrier selected from the group consisting of a nucleic acid sequence according to Claim 23 and a recombinant DNA molecule selected from the group consisting of a cDNA comprising a nucleic acid sequence according to Claim 23 and a nucleic acid sequence according to Claim 23, under the control of a functionally linked promoter; and, a host cell comprising a sequence selected from the group consisting of a nucleic acid sequence according to Claim 23, a cDNA fragment comprising a

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nucleic acid sequence according to Claim 23, a recombinant DNA molecule comprising a sequence selected from the group consisting of a nucleic acid sequence according to Claim 23 and a cDNA comprising a nucleic acid sequence according to Claim 23, under the control of a functionally linked promoter, and, a live recombinant carrier comprising a carrier selected from the group consisting of a nucleic acid sequence according to Claim 23 and a recombinant DNA molecule comprising a sequence selected from the group consisting of a nucleic acid sequence according to Claim 23 and a cDNA comprising a nucleic acid sequence according to Claim 23, under the control of a functionally linked promoter.

37. The vaccine of claim 36 further comprising an adjuvant.
38. The vaccine of claim 36 further comprising an additional antigen derived from a virus or microorganism pathogenic to dogs or a nucleic acid sequence encoding said antigen.
39. The vaccine according to claim 38, wherein said virus or micro-organism pathogenic to dogs is selected from the group of *Ehrlichia canis*, *Babesia gibsoni*, *vogeli*, *rossi*, *Leishmania donovani*-complex, Canine parvovirus, Canine distempervirus, *Leptospira interrogans* serovar *canicola*, *icterohaemorrhagiae*, *pomona*, *grippotyphosa*, *bratislava*, Canine hepatitisvirus, Canine parainfluenzavirus, rabies virus, *Hepatozoon canis* and *Borrelia burgdorferi*.

40. A vaccine for combating *Babesia canis* infections, comprising antibodies against a protein selected from the group consisting of at least one of the proteins of claims 32-35, or immunogenic fragment thereof.

41. A diagnostic test for the detection of *Babesia canis* associated RNA wherein the test comprises a nucleic acid sequence according to Claim 23, a nucleotide sequence that is complementary to said nucleic acid sequence, and a fragment thereof having a length of at least 12 nucleotides.

42. A diagnostic test for the detection of antibodies against *Babesia canis* associated antigenic material, wherein said test comprises a protein or an immunogenic fragment thereof as defined in claims 32-35.

43. A diagnostic test for the detection of *Babesia canis* associated antigenic material, wherein said test comprises antibodies against a protein or an immunogenic fragment thereof as defined in claims 32-35.

44. A nucleic acid sequence encoding a 32 kD *Babesia canis* associated protein or an immunogenic fragment of said protein, said protein or immunogenic fragment thereof having at least 80% homology with the amino acid sequence as depicted in SEQ ID NO:4.

45. The nucleic acid sequence of Claim 44 having at least 90% homology with the amino acid sequence as depicted in SEQ ID NO:4.

46. The nucleic acid sequence of Claim 44 having at least 95% homology with the amino acid sequence as depicted in SEQ ID NO:4.

47. A cDNA comprising a nucleic acid sequence according to Claim 44.

48. A recombinant DNA molecule comprising a nucleic acid sequence according to Claim 44, under the control of a functionally linked promoter.

49. A recombinant DNA molecule comprising a cDNA comprising a nucleic acid sequence according to Claim 44, under the control of a functionally linked promoter.

50. A live recombinant carrier comprising a cDNA comprising a nucleic acid sequence according to Claim 44.

51. A live recombinant carrier comprising a recombinant DNA molecule selected from the group consisting of a nucleic acid sequence according to Claim 44 and a cDNA comprising a nucleic acid sequence according to Claim 44, under the control of a functionally linked promoter.

52. A host cell comprising sequence selected from the group consisting of a nucleic acid sequence according to Claim 44; a cDNA fragment comprising a nucleic acid sequence according to Claim 44; a recombinant DNA molecule comprising a sequence selected from the group consisting of a nucleic acid sequence according to Claim 44 and a cDNA comprising a nucleic acid sequence according to Claim 44, under the control of a functionally linked promoter; and, a live

recombinant carrier comprising a carrier selected from the group consisting of a nucleic acid sequence according to Claim 44 and a recombinant DNA molecule comprising a sequence selected from the group consisting of a nucleic acid sequence according to Claim 44 and a cDNA comprising a nucleic acid sequence according to Claim 44, under the control of a functionally linked promoter.

53. A *Babesia canis* associated protein, said protein having a molecular weight of 32 kD and comprising an amino acid sequence that is at least 80% homologous to the amino acid sequence as depicted in SEQ ID NO:4 or an immunogenic fragment of said protein.

54. The *Babesia canis* associated protein of claim 53 wherein the amino acid sequence is at least 85% homologous to the amino acid sequence as depicted in SEQ ID NO: 4, or an immunogenic fragment of said protein.

55. The *Babesia canis* associated protein of claim 53 wherein the amino acid sequence is at least 90% homologous to the amino acid sequence as depicted in SEQ ID NO: 4, or an immunogenic fragment of said protein.

56. The *Babesia canis* associated protein of claim 53 wherein the amino acid sequence is at least 95% homologous to the amino acid sequence as depicted in SEQ ID NO: 4, or an immunogenic fragment of said protein.

57. A vaccine for combating *Babesia canis* infections, comprising an immunogen selected from the group consisting of a nucleic acid sequence according to Claim 44; a cDNA

fragment comprising a nucleic acid sequence according to Claim 44; a recombinant DNA molecule comprising a sequence selected from the group consisting of a nucleic acid sequence according to Claim 44 and a cDNA comprising a nucleic acid according to Claim 44, under the control of a functionally linked promoter; a live recombinant carrier selected from the group consisting of a nucleic acid sequence according to Claim 44 and a recombinant DNA molecule selected from the group consisting of a cDNA comprising a nucleic acid sequence according to Claim 44 and a nucleic acid sequence according to Claim 44, under the control of a functionally linked promoter; and, a host cell comprising sequence selected from the group consisting of a nucleic acid sequence according to Claim 44, a cDNA fragment comprising a nucleic acid sequence according to Claim 44, a recombinant DNA molecule comprising a sequence selected from the group consisting of a nucleic acid sequence according to Claim 44 and a cDNA comprising a nucleic acid sequence according to Claim 44, under the control of a functionally linked promoter, and, a live recombinant carrier comprising a carrier selected from the group consisting of a nucleic acid sequence according to Claim 44 and a recombinant DNA molecule comprising a sequence selected from the group consisting of a nucleic acid sequence according to Claim 44 and a cDNA comprising a nucleic acid sequence according to Claim 44, under the control of a functionally linked promoter.

58. The vaccine of claim 57 further comprising an adjuvant.

59. The vaccine of claim 57 further comprising an additional antigen derived from a virus or microorganism pathogenic to dogs or a nucleic acid sequence encoding said antigen.

60. The vaccine according to claim 59, wherein said virus or micro-organism pathogenic to dogs is selected from the group of *Ehrlichia canis*, *Babesia gibsoni*, *vogeli*, *rossi*, *Leishmania donovani*-complex, Canine parvovirus, Canine distempervirus, *Leptospira interrogans* serovar *canicola*, *icterohaemorrhagiae*, *pomona*, *grippotyphosa*, *bratislava*, Canine hepatitisvirus, Canine parainfluenzavirus, rabies virus, *Hepatozoon canis* and *Borrelia burgdorferi*.

61. A vaccine for combating *Babesia canis* infections, comprising antibodies against a protein selected from the group consisting of at least one of the proteins of Claims 53-56, or immunogenic fragment thereof.

62. A diagnostic test for the detection of antibodies against *Babesia canis* associated antigenic material, wherein said test comprises a protein or an immunogenic fragment thereof as defined in claims 52-55

63. A diagnostic test for the detection of *Babesia canis* associated antigenic material, wherein said test comprises antibodies against a protein or an immunogenic fragment thereof as defined in claims 52-55. - -

New claims 23-63 are original claims 1-22 re-written in a format proper for U.S. examination. The amendments made were not based on reasons related to patentability under 35 U.S.C. §§ USC 101, 102, 103 and/or 112. No estoppel should result from said amendments.

**III. In the Specification (Clean Sheet)**

(Page 1 lines 2-14)

**Field of the Invention**

The present invention relates to nucleic acid sequences encoding novel *Babesia canis* associated proteins, to cDNA fragments, recombinant DNA molecules and live recombinant carriers comprising these sequences and to host cells comprising such nucleic acid sequences, cDNA fragments, recombinant DNA molecules and live recombinant carriers. Furthermore, the invention relates to proteins encoded by these nucleotide sequences, to vaccines for combating *Babesia canis* infections and methods for the preparation thereof, to *Babesia canis* associated antigenic material for use in vaccines and to the use of *Babesia canis* associated antigenic material in the manufacture of vaccines. Also, the invention relates to diagnostic tools for the detection of *Babesia canis* associated nucleic acid sequences, for the detection of *Babesia canis* associated antigens and for the detection of antibodies against *Babesia canis* associated antigenic material.

(Page 1 line 15-23)

**Background**

Babesiosis, like malaria, is a disease which has a focal character. The reason for this is that the pathogen is transmitted by ticks that feed on a certain reservoir of parasites present in the vertebrate population. Only where ticks are present, babesiosis can occur. On balance, particularly in indigenous animals, the parasite coexists with the host without causing significant disease. In many

cases babesiosis is a problem because of man's activities through inbreeding of genetic traits and/or transporting animals to unfamiliar environments where babesiosis is endemic (Callow, L.L and Dalgliesh, R.J., 1982).

(Page 3 lines 3-8)

## Summary

It is i.a. an objective of the present invention to provide *Babesia canis* related proteins which are able, each separately, or when combined, to induce protection against *Babesia canis* infection in dogs. It was surprisingly found now, that two such novel proteins exist that are specifically associated with *Babesia canis*: they are not found in the *Babesia* species *B. rossi*.

(Page 3 lines 25-31)

## Detailed Description

It is well-known in the art, that many different nucleic acid sequences can encode one and the same protein. This phenomenon is commonly known as wobble in the second and especially the third base of each triplet encoding an amino acid. This phenomenon can result in heterology of about 30% for two different nucleic acid sequences still encoding the same protein. Therefore, two nucleic acid sequences having a sequence homology of about 70% can still encode one in the same protein.

**IV. In the Specification (Marked Version)**

Please insert -- **Field of the Invention** -- on page 1 line 2.

Please insert -- **Background** -- at page 1 line 15.

Please insert -- **Summary** -- on page 3 line 3.

Please insert -- **Detailed Description** -- at page 3 line 25.

**V. Remarks**

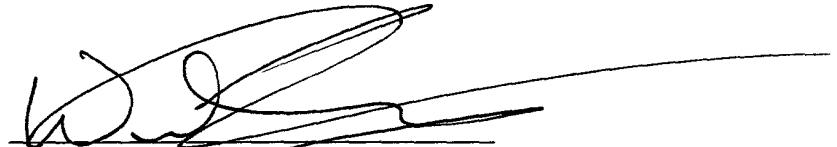
Applicants' US filing is a duplicate copy of the originally filed European patent application. Applicants have preliminarily amended the claims to the originally filed European patent application to place the claims in proper condition for U.S examination. The amendments to the claims and the specification were not made for reasons of patentability under 35 U.S.C. §§ 101, 102, 103 and/or 112. No estoppel should result from the amendments.

**VI. Conclusion**

Applicants respectfully file this application. Applicants believe the application is in a condition for allowance and respectfully request such action. Applicants further

respectfully request the Examiner call the undersigned attorney  
to assist the application in securing allowance.

Respectfully Submitted,



William P. Ramey III  
Patent Counsel  
Registration Number 44,295

Attorney Docket Number I 2001.004 US

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*Babesia canis* vaccine.

The present invention relates to nucleic acid sequences encoding novel *Babesia canis* associated proteins, to cDNA fragments, recombinant DNA molecules and live  
5 recombinant carriers comprising these sequences and to host cells comprising such nucleic acid sequences, cDNA fragments, recombinant DNA molecules and live recombinant carriers. Furthermore, the invention relates to proteins encoded by these nucleotide sequences, to vaccines for combating *Babesia canis* infections and methods for the preparation thereof, to *Babesia canis* associated antigenic material for  
10 use in vaccines and to the use of *Babesia canis* associated antigenic material in the manufacture of vaccines. Also, the invention relates to diagnostic tools for the detection of *Babesia canis* associated nucleic acid sequences, for the detection of *Babesia canis* associated antigens and for the detection of antibodies against *Babesia canis* associated antigenic material.  
15  
Babesiosis, like malaria, is a disease which has a focal character. The reason for this is that the pathogen is transmitted by ticks that feed on a certain reservoir of parasites present in the vertebrate population. Only where ticks are present, babesiosis can occur. On balance, particularly in indigenous animals, the parasite coexists with the  
20 host without causing significant disease. In many cases babesiosis is a problem because of man's activities through inbreeding of genetic traits and/or transporting animals to unfamiliar environments where babesiosis is endemic (Callow, L.L and Dalgliesh, R.J., 1982).  
In dogs the disease is caused by *B. canis* (transmitted by Dermacentor ticks), *B. rossi*  
25 (transmitted by Haemaphysalis ticks), *B. vogeli* (transmitted by Rhipicephalus ticks) and *Babesia gibsoni* (transmitted by Haemaphysalis and Rhipicephalus ticks). Signs of disease in naturally acquired babesiosis usually begin 7-21 days after infection. These symptoms include: fever, anorexia, depression, anaemia, haemoglobinuria and rapidly developing weakness. Increased lacrimation, salivation  
30 and muscle tremor commonly occur. Nervous signs may develop in terminal infections, and death may occur when the disease is left untreated. Severe coagulation disturbances resembling disseminated intravascular coagulation (DIC) have been reported in acute *B. canis* infections. Thrombosis is not common, but small hyaline thrombi, connected with megakaryocytes have been described. It appears that these

coagulation disturbances lead to increased erythrocyte-stickiness. As a result the blood passage through the microvasculature is hampered, resulting in congestion of internal organs and decreased packed cell volumes (PCV). This might impair the oxygen supply to certain tissues and subsequently lead to tissue damage as a result of 5 anoxia. Evidence from congestion in *B. canis* infections comes from experiments in which dogs were chemotherapeutically treated. Some of these animals restore the packed cell volume in two days from 25-35%, which is associated with shrinkage of the spleen to normal measures.

The four species of *Babesia* differ with respect to their pathogenicity. The

10 North-African *B. vogeli* strains provoke only mild disease, which usually does not require treatment (like the Australian *B. canis* strains). European *B. canis* strains are more pathogenic than the North-African parasite. In our experiments with the *B. canis* A strain, approximately half of the animals required chemotherapeutic treatment after infection. The South-African *B. rossi* strains are most pathogenic, a feature observed 15 already very early. Using the South-African *B. rossi* strain naive dogs developed progressing disease characterised by exponential parasite growth. In contrast, parasitaemia in dogs infected with European *B. canis* usually is limited. In the latter, congestion appears to be the main pathological feature.

20 At the moment a vaccine for *Babesia* is prepared from the supernatant of a culture of a strain of *Babesia canis*, as is described in US Patent 4,777,036. However, such a vaccine contains only antigens of the strain of this particular species of *B. canis*. It appears that such a vaccine gives in general little protection against infections with (wild type) *B. canis* (Lepetit, C., Piroplasmose canine et vaccination Pirodog, 25 Doctoral Thesis, Univ. of Nantes, 1988). The author of the thesis gives three possible causes of this failure of protective immunisation:

1. the sensitivity of the dogs with respect to vaccination;
2. the type of immune effector mechanism induced by vaccination;
3. the antigenic diversity of *Babesia*.

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Vaccines containing whole, attenuated *Babesia* parasites always harbour the danger that the parasites again become virulent and thus spread the disease instead of curing it. Therefore, the use of subunit vaccines is preferred. Thus there remains the desire for a vaccine which is not infective, which can be easily produced and which yet can

give protection against *Babesia canis* infection, preferably against all *Babesia canis* strains.

It is i.a. an objective of the present invention to provide *Babesia canis* related proteins

5 which are able, each separately, or when combined, to induce protection against *Babesia canis* infection in dogs. It was surprisingly found now, that two such novel proteins exist that are specifically associated with *Babesia canis*: they are not found in the *Babesia* species *B. rossi*.

10 By referring to the novel proteins as associated with *Babesia canis* it is meant that these proteins are found to be present in the *Babesia canis* parasite. It does not mean that the genetic information is found on a chromosome of the parasite. The genetic information for the novel proteins is found in the parasite, but it can be present as extra-chromosomal information.

15 The novel proteins will be referred to as the 15 kD protein (also Bcvir15) and the 32 kD protein (also Bcvir32). The amino acid sequence of the 15 kD protein is presented in sequence identifier SEQ ID NO: 2. The cDNA encoding this protein has been sequenced and its nucleic acid sequence is shown in sequence identifier SEQ ID NO:

20 1.

The amino acid sequence of the 32 kD protein is presented in sequence identifier SEQ ID NO: 4. The cDNA encoding this protein has been sequenced and its nucleic acid sequence is shown in sequence identifier SEQ ID NO: 3.

25 It is well-known in the art, that many different nucleic acid sequences can encode one and the same protein. This phenomenon is commonly known as wobble in the second and especially the third base of each triplet encoding an amino acid. This phenomenon can result in a heterology of about 30% for two different nucleic acid sequences still 30 encoding the same protein. Therefore, two nucleic acid sequences having a sequence homology of about 70 % can still encode one and the same protein.

One embodiment of the invention relates to a nucleic acid sequence encoding a 15 kD *Babesia canis* associated protein or an immunogenic fragment of that protein, said

protein or immunogenic fragment thereof having at least 80 %, preferably 90 %, more preferably 95 % homology with the amino acid sequence as depicted in SEQ ID NO:

2. Even more preferred is a homology level of 98% or even 100%.

5 Another embodiment of the invention relates to a nucleic acid sequence encoding a 32 kD *Babesia canis* associated protein or an immunogenic fragment of that protein, said protein or immunogenic fragment thereof having at least 80 %, preferably 90 %, more preferably 95 % homology with the amino acid sequence as depicted in SEQ ID NO:

4. Even more preferred is a homology level of 98% or even 100%.

10

Needless to say that a nucleic acid sequence encoding a protein according to the invention is understood to apply equally to the RNA encoding the protein and to cDNA and DNA carrying the genetic code of this RNA. As a consequence, where a certain nucleic acid sequence or part thereof is said to have a certain level of

15 homology with e.g. the nucleic acid sequence of SEQ ID NO: 1, this level of homology equally goes for cDNA and the RNA used as a template for making the cDNA, as well as vice versa to (c)DNA and the RNA transcribed from that DNA.

Parts of the said nucleic acid sequences that encode an immunogenic fragment of the  
20 proteins to which the invention relates can be very small. Immunogenic fragments of e.g. 4 amino acid length are known. Such immunogenic fragments are encoded by a nucleic acid sequence of 12 nucleotides. Such very short amino acid sequences are however not immunogenic as such: they need to be coupled to a carrier, such as KHL, as known in the art. Longer fragments can be immunogenic as such. Therefore, the  
25 parts of the said nucleic acid sequences that encode an immunogenic fragment comprise at least 12 nucleotides. Parts comprising more than 15, more preferably more than 18, 20 25 or even 30 nucleotides are even more preferred in that order of preference.

30 Since the present invention discloses nucleic acid sequences encoding novel *Babesia canis* associated 15 kD and 32 kD proteins, it is now for the first time possible to obtain these proteins in sufficient quantities. This can e.g. be done by using expression systems to express the genes encoding the proteins.

PCT/EP2007/000001

Therefore, in another embodiment, the invention relates to a cDNA fragment comprising a nucleic acid sequence according to the invention. A cDNA is a copy DNA that has been made, e.g. by reverse transcriptase, of an RNA encoding a protein according to the invention. Such cDNA fragments in their shortest form are a DNA 5 copy of a part of a nucleotide sequence according to the invention. They can however also comprise flanking nucleic acid sequences not naturally present upstream and downstream of the coding sequence.

An essential requirement for the expression of cDNA is an adequate promoter

10 functionally linked to the cDNA, so that the cDNA is under the control of the promoter. It is obvious to those skilled in the art that the choice of a promoter extends to any eukaryotic, prokaryotic or viral promoter capable of directing gene transcription in cells used as host cells for protein expression.

Therefore, another embodiment of the invention relates to a recombinant DNA

15 molecule comprising a cDNA fragment or a nucleic acid sequence according to the invention that is placed under the control of a functionally linked promoter. This can be obtained by means of e.g. standard molecular biology techniques.

(Maniatis/Sambrook (Sambrook, J. Molecular cloning: a laboratory manual, 1989. ISBN 0-87969-309-6).

20 Functionally linked promoters are promoters that are capable of controlling the transcription of the nucleic acid sequences to which they are linked.

Such a promoter can be a *Babesia* promoter, provided that that promoter is functional in the cell used for expression. It can also be a heterologous promoter. When the host cells are bacteria, useful expression control sequences which may be used include the

25 Trp promoter and operator (Goeddel, et al., Nucl. Acids Res., 8, 4057, 1980); the lac promoter and operator (Chang, et al., Nature, 275, 615, 1978); the outer membrane protein promoter (Nakamura, K. and Inouge, M., EMBO J., 1, 771-775, 1982); the bacteriophage lambda promoters and operators (Remaut, E. et al., Nucl. Acids Res., 11, 4677-4688, 1983); the  $\alpha$ -amylase (*B. subtilis*) promoter and operator, termination 30 sequences and other expression enhancement and control sequences compatible with the selected host cell.

When the host cell is yeast, useful expression control sequences include, e.g.,  $\alpha$ -mating factor. For insect cells the polyhedrin or p10 promoters of baculoviruses can

be used (Smith, G.E. et al., Mol. Cell. Biol. 3, 2156-65, 1983). When the host cell is of mammalian origin illustrative useful expression control sequences include the SV-40 promoter (Berman, P.W. et al., Science, 222, 524-527, 1983) or the metallothionein promoter (Brinster, R.L., Nature, 296, 39-42, 1982) or a heat shock

5 promoter (Voellmy et al., Proc. Natl. Acad. Sci. USA, 82, 4949-53, 1985).

Bacterial, yeast, fungal, insect and mammalian cell expression systems are very frequently used systems. Such systems are well-known in the art and generally available, e.g. commercially through Clontech Laboratories, Inc. 4030 Fabian Way,

10 Palo Alto, California 94303-4607, USA. Next to these expression systems, parasite-based expression systems are very attractive expression systems. Such systems are e.g. described in the French Patent Application with Publication number 2 714 074, and in US NTIS Publication No US 08/043109 (Hoffman, S. and Rogers, W.: Public. Date 1 December 1993).

15

Another embodiment of the invention relates to a Live Recombinant Carrier (LRC) comprising a cDNA fragment according to the invention or a recombinant DNA molecule according to the invention. Such carriers are e.g. bacteria, parasites and viruses. These LRCs are micro-organisms or viruses in which additional genetic

20 information, in this case a nucleic acid sequence encoding the 15 kD or 32 kD protein or an immunogenic fragment thereof according to the invention has been cloned. Animals infected with such LRCs will produce an immunogenic response not only against the immunogens of the carrier, but also against the immunogenic parts of the protein(s) for which the genetic code is additionally cloned into the LRC, e.g. the 15

25 kD or 32 kD cDNA.

As an example of bacterial LRCs, attenuated *Salmonella* strains known in the art can attractively be used.

Live recombinant carrier parasites have i.a. been described by Vermeulen, A. N. (Int. Journ. Parasitol. 28: 1121-1130 (1998)).

30 Also, LRC viruses may be used as a way of transporting the nucleic acid sequence into a target cell. Live recombinant carrier viruses are also called vector viruses. Viruses often used as vectors are Vaccinia viruses (Panicali et al; Proc. Natl. Acad. Sci. USA, 79: 4927 (1982), Herpesviruses (E.P.A. 0473210A2), and Retroviruses (Valerio, D. et al; in Baum, S.J., Dicke, K.A., Lotzova, E. and Pluznik, D.H. (Eds.),

Experimental Haematology today - 1988. Springer Verlag, New York: pp. 92-99  
(1989)).

The technique of *in vivo* homologous recombination, well-known in the art, can be  
5 used to introduce a recombinant nucleic acid sequence into the genome of a  
bacterium, parasite or virus of choice, capable of inducing expression of the inserted  
nucleic acid sequence according to the invention in the host animal.

Finally another embodiment of the invention relates to a host cell comprising a  
10 nucleic acid sequence according to the invention, a cDNA fragment or a recombinant  
DNA molecule according to the invention or a live recombinant carrier according to  
the invention. A host cell may be a cell of bacterial origin, e.g. *Escherichia coli*,  
*Bacillus subtilis* and *Lactobacillus* species, in combination with bacteria-based  
plasmids as pBR322, or bacterial expression vectors as pGEX, or with

15 bacteriophages. The host cell may also be of eukaryotic origin, e.g. yeast-cells in  
combination with yeast-specific vector molecules, or higher eukaryotic cells like  
insect cells (Luckow et al; Bio-technology 6: 47-55 (1988)) in combination with  
vectors or recombinant baculoviruses, plant cells in combination with e.g. Ti-plasmid  
based vectors or plant viral vectors (Barton, K.A. et al; Cell 32: 1033 (1983)),  
20 mammalian cells like Hela cells, Chinese Hamster Ovary cells (CHO) or Crandell  
Feline Kidney-cells, also with appropriate vectors or recombinant viruses.

Another embodiment of the invention relates to the novel *Babesia canis* associated  
proteins: the 15 kD and 32 kD protein and to immunogenic fragments thereof,  
25 according to the invention.

The concept of immunogenic fragments will be defined below.

It will be understood that, for the particular proteins embraced herein, natural  
variations can exist between the proteins associated with individual *Babesia canis*  
strains. These variations may be demonstrated by (an) amino acid difference(s) in the  
30 overall sequence or by deletions, substitutions, insertions, inversions or additions of  
(an) amino acid(s) in said sequence. Amino acid substitutions which do not essentially  
alter biological and immunological activities, have been described, e.g. by Neurath et  
al in "The Proteins" Academic Press New York (1979). Amino acid replacements  
between related amino acids or replacements which have occurred frequently in

evolution are, inter alia, Ser/Ala, Ser/Gly, Asp/Gly, Asp/Asn, Ile/Val (see Dayhof, M.D., Atlas of protein sequence and structure, Nat. Biomed. Res. Found., Washington D.C., 1978, vol. 5, suppl. 3). Other amino acid substitutions include Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Thr/Phe, Ala/Pro, Lys/Arg, Leu/Ile, Leu/Val and

5 Ala/Glu. Based on this information, Lipman and Pearson developed a method for rapid and sensitive protein comparison (Science, 227, 1435-1441, 1985) and determining the functional similarity between homologous proteins. Such amino acid substitutions of the exemplary embodiments of this invention, as well as variations having deletions and/or insertions are within the scope of the invention as long as the resulting proteins retain immune reactivity.

10 This explains why *Babesia canis* associated proteins according to the invention, when isolated from different field isolates, may have homology levels of about 80%, while still representing the same protein with the same immunological characteristics, i.e. the capability of inducing an immunological response against a *Babesia canis* infection.

15 Those variations in the amino acid sequence of a certain protein according to the invention that still provide a protein capable of inducing an immune response against infection with *Babesia canis* or at least against the clinical manifestations of the infection are considered as "not essentially influencing the immunogenicity".

20 Therefore, one form of this embodiment relates i.a. to a *Babesia canis* associated protein that has a molecular weight of 15 kD and comprises an amino acid sequence that is at least 80 % homologous to the amino acid sequence as depicted in SEQ ID NO: 2 and to an immunogenic fragment of said protein.

25 In a preferred form, the embodiment relates to such a *Babesia canis* associated protein that has a sequence homology of at least 85 %, preferably 90 %, more preferably 95 % homology to the amino acid sequence as depicted in SEQ ID NO: 2 and to an immunogenic fragment of such a protein.

30 Even more preferred is a homology level of 98% or even 100%.

Another embodiment relates i.a. to a *Babesia canis* associated protein that has a molecular weight of 32 kD and comprises an amino acid sequence that is at least 80 % homologous to the amino acid sequence as depicted in SEQ ID NO: 4 and to an

immunogenic fragment of said protein.

A preferred form of this embodiment relates to such a *Babesia canis* associated protein that has a sequence homology of at least 85 %, preferably 90 %, more

5 preferably 95 % homology to the amino acid sequence as depicted in SEQ ID NO: 4  
and to an immunogenic fragment of such a protein.

Even more preferred is a homology level of 98% or even 100%.

The level of protein homology can be determined with the computer program

10 "BLAST 2 SEQUENCES" by selecting sub-program: "BLASTP", that can be found at [www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html](http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html).

A reference for this program is Tatiana A. Tatusova, Thomas L. Madden FEMS Microbiol. Letters 174: 247-250 (1999). Matrix used: "blosum62". Parameters used are the default parameters: Open gap: 11. Extension gap: 1. Gap x\_dropoff: 50.

15

When a protein is used for e.g. vaccination purposes or for raising antibodies, it is however not necessary to use the whole protein. It is also possible to use a fragment of that protein that is capable, as such or coupled to a carrier such as e.g. KLH, of inducing an immune response against that protein, a so-called immunogenic fragment.

20 An “immunogenic fragment” is understood to be a fragment of the full-length protein that still has retained its capability to induce an immune response in the host, i.e. comprises a B- or T-cell epitope. At this moment, a variety of techniques is available to easily identify cDNA fragments encoding antigenic fragments (determinants). The method described by Geysen et al (Patent Application WO 84/03564, Patent

25 Application WO 86/06487, US Patent NR. 4,833,092, Proc. Natl Acad. Sci. 81: 3998-  
4002 (1984), J. Imm. Meth. 102, 259-274 (1987)), the so-called PEPSCAN method is  
an easy to perform, quick and well-established method for the detection of epitopes,  
the immunologically important regions of the protein. The method is used world-wide  
and as such well-known to man skilled in the art. This (empirical) method is

especially suitable for the detection of B-cell epitopes. Also, given the sequence of the gene encoding any protein, computer algorithms are able to designate specific protein fragments as the immunologically important epitopes on the basis of their sequential and/or structural agreement with epitopes that are now known. The determination of these regions is based on a combination of the hydrophilicity criteria according to

Hopp and Woods (Proc. Natl. Acad. Sci. 78: 38248-3828 (1981)), and the secondary structure aspects according to Chou and Fasman (Advances in Enzymology 47: 45-148 (1987) and US Patent 4,554,101). T-cell epitopes can likewise be predicted from the sequence by computer with the aid of Berzofsky's amphiphilicity criterion

5 (Science 235, 1059-1062 (1987) and US Patent application NTIS US 07/005,885). A condensed overview is found in: Shan Lu on common principles: Tibtech 9: 238-242 (1991), Good et al on Malaria epitopes; Science 235: 1059-1062 (1987), Lu for a review; Vaccine 10: 3-7 (1992), Berzowsky for HIV-epitopes; The FASEB Journal 5:2412-2418 (1991).

10

Thus, another embodiment of the invention relates to a vaccine for combating *Babesia canis* infection, that comprises a protein or an immunogenic fragment thereof according to the invention as described above together with a pharmaceutically acceptable carrier.

15

One way of making a vaccine according to the invention comprises growing the *Babesia canis* parasite, and purification of the 15 kD and 32 kD proteins. This is however a very time-consuming way of making the vaccine. It would thus be less practical to make such vaccines on an industrial scale.

20

It is one of the merits of the present invention that it provides for the first time the nucleic acid sequences encoding the 15 kD and 32 kD proteins. It is therefore much more convenient to use the expression products of the cDNA encoding these proteins or immunogenic fragments thereof according to the invention in vaccines.

25

Vaccines based upon the expression products of these cDNAs can easily be made by admixing one or both proteins according to the invention or immunogenic fragments thereof according to the invention with a pharmaceutically acceptable carrier as described below. It is also possible to directly admix a host cell in which one or both 30 proteins according to the invention or immunogenic fragments thereof according to the invention have been expressed and a pharmaceutically acceptable carrier.

Alternatively, a vaccine according to the invention can comprise live recombinant carriers as described above, capable of expressing the proteins according to the

invention or immunogenic fragments thereof according to the invention. Such vaccines, e.g. based upon a bacterial (e.g. *Salmonella*) carrier, a parasitic or a viral carrier have the advantage over subunit vaccines that they better mimic the natural way of infection of *Babesia canis*. Moreover, their self-propagation is an advantage  
5 since only low amounts of the recombinant carrier are necessary for immunisation. Thus, still another embodiment relates to a vaccine for combating *Babesia canis* infection, that comprises a live recombinant carrier according to the invention and a pharmaceutically acceptable carrier.

10 All vaccines described above contribute to active vaccination, i.e. the host's immune system is triggered by one or more proteins according to the invention or immunogenic fragments thereof, to make antibodies against these proteins or fragments or to expand T-cell populations with receptors that can be specifically triggered by epitopes associated with these proteins.

15 Alternatively, such antibodies can be raised in e.g. rabbits or can be obtained from antibody-producing cell lines as described below. Such antibodies can then be administered to the host animal. This method of vaccination, passive vaccination, is the vaccination of choice when an animal is already infected, and there is no time to allow the natural immune response to be triggered. It is also the preferred method for  
20 vaccinating immune-compromised animals. Administered antibodies against *Babesia canis* can in these cases bind directly to the proteins according to the invention and to parasites and cells exposing these proteins. This has the advantage that it decreases or stops *Babesia canis* growth.

Therefore, one other form of this embodiment of the invention relates to a vaccine for  
25 combating *Babesia canis* infection comprising antibodies against a *Babesia canis* associated protein according to the invention or an immunogenic fragment of those proteins, and a pharmaceutically acceptable carrier.

Another embodiment relates to a method for the preparation of a vaccine according to  
30 the invention that comprises the admixing of antibodies according to the invention and a pharmaceutically acceptable carrier.

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Vaccines can also be based upon host cells as described above, that comprise the nucleic acid sequences, cDNA fragments, recombinant DNA molecules or live recombinant carriers according to the invention.

Therefore, another embodiment of the present invention relates to a vaccine for combating *Babesia canis* infection, that comprises a host cell according to the invention and a pharmaceutically acceptable carrier.

An alternative and efficient way of vaccination is direct vaccination with DNA encoding the relevant antigen. Direct vaccination with DNA encoding proteins has been successful for many different proteins (As reviewed in e.g. Donnelly et al., *The Immunologist* 2: 20-26 (1993)).

This way of vaccination is also attractive for the vaccination of dogs against *Babesia canis* infection.

Therefore, still other forms of this embodiment of the invention relate to a vaccine

15 comprising a nucleic acid sequence according to the invention and a pharmaceutically acceptable carrier, and to a vaccine comprising a cDNA fragment according to the invention and a pharmaceutically acceptable carrier.

Still another form of this embodiment relates to a vaccine comprising a recombinant DNA molecule according to the invention and a pharmaceutically acceptable carrier.

20 DNA vaccines can easily be administered through intradermal application e.g. using a needle-less injector. This way of administration delivers the DNA directly into the cells of the animal to be vaccinated. Amounts of DNA in the microgram range between 1 and 100 µg provide very good results.

25 In a preferred embodiment, the vaccine according to the present invention comprises an additional antigen derived from a micro-organism or virus pathogenic to dogs, or genetic information encoding such an antigen.

Such dog pathogenic organisms and viruses are preferably selected from the group of *Ehrlichia canis*, *Babesia gibsoni*, *vogeli*, *rossi*, *Leishmania donovani*-complex,

30 Canine parvovirus, Canine distempervirus, *Leptospira interrogans* serovar *canicola*,  
*icterohaemorrhagiae*, *pomona*, *grippotyphosa*, *bratislava*, Canine hepatitisvirus,  
Canine parainfluenzavirus, rabies virus, *Hepatozoon canis* and *Borrelia burgdorferi*.

All vaccines according to the present invention comprise a pharmaceutically acceptable carrier. A pharmaceutically acceptable carrier can be e.g. sterile water or a sterile physiological salt solution. In a more complex form the carrier can e.g. be a buffer.

5

Methods for the preparation of a vaccine comprise the admixing of a nucleic acid sequence, a cDNA fragment, a recombinant DNA molecule or a live recombinant carrier according to according to the invention, a host cell according to the invention, or a protein according to the invention or an immunogenic fragment thereof and a pharmaceutically acceptable carrier.

10

Thus, another embodiment of the present invention relates to a method for the preparation of a vaccine according to the invention, that comprises the admixing of a nucleic acid sequence according to the invention, a cDNA fragment the invention, a recombinant DNA molecule according to the invention, a live recombinant carrier according to the invention or a host cell according to the invention or a protein according to the invention and a pharmaceutically acceptable carrier.

15

Vaccines according to the present invention may in a preferred presentation also contain an adjuvant. Adjuvants in general comprise substances that boost the immune response of the host in a non-specific manner. A number of different adjuvants are known in the art. Examples of adjuvants are Freunds Complete and Incomplete adjuvant, vitamin E, non-ionic block polymers, muramyldipeptides, saponins such as Quill A(R), mineral oil e.g. Bayol(R) or Markol(R), vegetable oil, and Carbopol(R) (a homopolymer), or Diluvac(R) Forte.

25

The vaccine may also comprise a so-called "vehicle". A vehicle is a compound to which the protein adheres, without being covalently bound to it. Often used vehicle compounds are e.g. aluminium hydroxide, -phosphate or -oxide, silica, Kaolin, and Bentonite.

A special form of such a vehicle, in which the antigen is partially embedded in the vehicle, is the so-called ISCOM (EP 109.942, EP 180.564, EP 242.380)

In addition, the vaccine may comprise one or more suitable surface-active compounds or emulsifiers, e.g. Span or Tween.

Often, the vaccine is mixed with stabilisers, e.g. to protect degradation-prone proteins from being degraded, to enhance the shelf-life of the vaccine, or to improve freeze-drying efficiency. Useful stabilisers are i.a. SPGA (Bovarnik et al; J. Bacteriology 59: 509 (1950)), carbohydrates e.g. sorbitol, mannitol, trehalose, starch, sucrose, dextran or glucose, proteins such as albumin or casein or degradation products thereof, and buffers, such as alkali metal phosphates.

In addition, the vaccine may be suspended in a physiologically acceptable diluent.

It goes without saying, that other ways of adjuvating, adding vehicle compounds or diluents, emulsifying or stabilising a protein are also embodied in the present invention.

Thus, a preferred form of this embodiment relates to a vaccine for combating *Babesia canis* infections according to the invention that comprises an adjuvant.

Vaccines according to the invention can very suitably be administered in amounts ranging between 1 and 100 micrograms, although smaller doses can in principle be used. A dose exceeding 100 micrograms will, although immunologically very suitable, be less attractive for commercial reasons.

Vaccines based upon live attenuated recombinant carriers, such as the LRC-viruses, parasites and bacteria described above can be administered in much lower doses, because they multiply themselves during the infection. Therefore, suitable amounts would range between  $10^3$  and  $10^9$  CFU/PFU for respectively bacteria, parasites and viruses.

Many ways of administration can be applied. Systemic application is a suitable way of administration, e.g. by intramuscular application of the vaccine. If this route is followed, standard procedures known in the art for systemic application are well-suited. Intradermal, sub-cutaneous and intracutaneous ways of administration are also attractive.

Still another embodiment of the present invention relates to a *Babesia canis* associated protein according to the invention for use in a vaccine.

Still another embodiment of the present invention relates to the use of a *Babesia canis* associated protein according to the invention for the manufacturing of a vaccine for combating *Babesia canis* infections.

5 From a point of view of protection against disease, a quick and correct diagnosis of *Babesia canis* infection is important.  
Therefore it is another objective of this invention to provide diagnostic tools suitable for the detection of *Babesia canis* infection.

10 A diagnostic test for the detection of *Babesia canis* is e.g. based upon the reaction of RNA isolated from the animal to be tested, with specific probes or it is e.g. a RT-PCR test based upon the coding sequences for the 15 kD or the 32 kD proteins or based upon nucleic acid sequences that are complementary to those coding sequences. If nucleic acid molecules specific for the *Babesia canis* associated proteins according to 15 the invention are present in the animal, these will e.g. specifically bind to specific PCR-primers and will subsequently become amplified in RT-PCR-reaction. The PCR-reaction product can then easily be detected in DNA gel electrophoresis. RT-PCR reactions are well-known in the art (see reference below). The nucleic acid molecules can most easily be isolated from (the parasites present in) erythrocytes of the animal 20 to be tested. Standard PCR-textbooks give methods for determining the length of the primers for selective PCR-reactions with nucleic acid molecules specific for the *Babesia canis* associated proteins according to the invention. Primers with a nucleotide sequence of at least 12 nucleotides are frequently used, but primers of more than 15, more preferably 18 nucleotides are somewhat more selective.  
25 Especially primers with a length of at least 20, preferably at least 30 nucleotides are very generally applicable. PCR-techniques are extensively described in (Dieffenbach & Dreksler; PCR primers, a laboratory manual. ISBN 0-87969-447-5 (1995)). Nucleic acid molecules encoding a *Babesia canis* associated protein or parts of those nucleic acid molecules having a length of at least 12, preferably 15, more preferably 30 18, even more preferably 20, 22, 25, 30, 35 or 40 nucleotides in that order of preference, wherein the nucleic acid molecules or parts hereof have at least 70 % homology with the nucleic acid sequence as depicted in SEQ ID NO: 1 or 3 or a nucleic acid sequence that is complementary to nucleic acid sequence as depicted in SEQ ID NO: 1 or 3 are therefore also part of the invention. Such nucleic acid

molecules can e.g. be used as primers in (RT-)PCR-reactions in order to enhance the amount of nucleic acid that encodes the proteins according to the invention. This allows the quick amplification of specific nucleotide sequences for use as a diagnostic tool for e.g. the detection of *Babesia* in tissue as indicated above.

5

The level of nucleotide homology can be determined with the computer program "BLAST 2 SEQUENCES" by selecting sub-program: "BLASTN" that can be found at [www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html](http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html).

A reference for this program is Tatiana A. Tatusova, Thomas J. Madden FEMS

10 Microbiol. Letters 174: 247-250 (1999). Parameters used are the default parameters:  
Reward for a match: +1. Penalty for a mismatch: -2. Open gap: 5. Extension gap: 2.  
Gap x dropoff: 50.

Another nuclei acid-based test is based upon growth of parasite material obtained

15 from erythrocytes, followed by classical RNA purification followed by cDNA synthesis and/or classical hybridisation with radioactively or colour labelled 15 kD or 32 kD protein-specific cDNA-fragments. Both PCR-reactions and hybridisation reactions are well-known in the art and are i.a. described in Maniatis/Sambrook (Sambrook, J. et al. Molecular cloning: a laboratory manual. ISBN 0-87969-309-6).

20

Thus, another embodiment of the invention relates to a diagnostic test for the detection of *Babesia canis* associated RNA characterised in that the test comprises a nucleic acid sequence that is at least 70 % homologous to the nucleic acid sequence as depicted in SEQ ID NO: 1 or 3 or a nucleotide sequence that is complementary to that nucleic acid sequence, or a fragment thereof having a length of at least 12, preferably 15, more preferably 18 nucleotides.

A diagnostic test for the detection of antibodies against the *Babesia canis* associated proteins in e.g. sera, tissue or body fluids can be e.g. a simple standard ELISA-test in which 15 kD or 32 kD protein or antigenic fragments thereof according to the invention are coated to the wall of the wells of an ELISA-plate. A method for the detection of such antibodies is e.g. incubation of 15 kD or 32 kD protein or antigenic fragments thereof with serum from mammals to be tested, followed by e.g. incubation with a labelled antibody against the relevant mammalian antibody. A colour reaction

can then reveal the presence or absence of antibodies against *Babesia canis* associated protein. Another example of a diagnostic test system is e.g. the incubation of a Western blot comprising the 15 kD or 32 kD protein or an antigenic fragment thereof according to the invention, with serum of mammals to be tested, followed by analysis  
5 of the blot.

Thus, another embodiment of the present invention relates to a diagnostic test for the detection of antibodies against *Babesia canis* associated antigenic material, characterised in that the test comprises a protein or an immunogenic fragment thereof  
10 according to the invention.

A diagnostic test based upon the detection of antigenic material of the specific 15 kD and 32 kD proteins amongst *Babesia canis* antigens and therefore suitable for the detection of *Babesia canis* infection can e.g. also be a standard sandwich-ELISA test.

15 In one example of such a test the walls of the wells of an ELISA plate are coated with antibodies directed against the 15 kD or 32 kD protein or immunogenic fragments thereof. After incubation with the material to be tested, labelled anti-*Babesia canis* antibodies are added to the wells. A colour reaction then reveals the presence of antigenic material from *Babesia canis*.

20 Therefore, still another embodiment of the present invention relates to a diagnostic test for the detection of *Babesia canis* associated antigenic material, characterised in that said test comprises antibodies against a protein or an immunogenic fragment thereof according to the invention.

25 The proteins or immunogenic fragments thereof according to the invention expressed as described above can be used to produce antibodies, which may be polyclonal, monospecific or monoclonal (or derivatives thereof). If polyclonal antibodies are desired, techniques for producing and processing polyclonal sera are well-known in the art (e.g. Mayer and Walter, eds. Immunochemical Methods in Cell and Molecular  
30 Biology, Academic Press, London, 1987). Monoclonal antibodies, reactive against the protein according to the invention (or variants or fragments thereof) according to the present invention, can be prepared by immunising inbred mice by techniques also known in the art (Kohler and Milstein, Nature, 256, 495-497, 1975).

Still another embodiment of the invention relates to methods for the detection of antigenic material from *Babesia canis* in which the method comprises the incubation of serum, tissue or body fluids with antibodies against the 15 kD or the 32 kD protein or an antigenic fragment thereof according to the invention.

## Examples

### EXAMPLE 1: Molecular characterisation of *BCVIR* cDNA

5    **Growth of *Babesia canis***

Isolates of *Babesia canis* (isolate A and B) were obtained from dogs naturally infected in France. They were maintained in *in vitro* culture according to Schetters *et al.* (1997, *Parasitology*, **115**, 485-493).

10    **cDNA library construction, immunological screening and isolation of plasmid DNA.**

A cDNA library from *Babesia canis* (isolate A) was constructed with the ZAP Express<sup>TM</sup> cDNA Gigapack<sup>R</sup> II Gold Cloning kit (Stratagene) as it was described for

*B. rossi* in Carret *et al.* (1999, *Eur. J. Biochem.*, **265**, 1015-1021). The library was

15    screened as described in Carret *et al.* (1999, *Eur. J. Biochem.*, **265**, 1015-1021) using the anti-GST60.1 (depleted of anti-*E. coli* antibodies), a polyclonal antibody directed against one member of the *Plasmodium falciparum* Pf60 multigene family (Carcy *et al.*, 1994, *Mol. Biochem. Parasitol.* **68**: 221-233), at a 1:100 dilution. Positive clones were visualised using goat anti-rabbit IgG conjugated to peroxidase (Sigma) at a

20    1:500 dilution, using 4-chloro-1-naphtol as chromogen (Sigma). They were purified and the pBK-CMV plasmids carrying the *BCVIR* cDNA were prepared as described in Carret *et al.* (1999, *Eur. J. Biochem.*, **265**, 1015-1021).

#### DNA sequencing

25    Nucleotid sequence was performed using the dideoxy chain termination method from alkali-denatured double-strand templates according to Sanger *et al.* (1977, *Proc. Natl. Acad. Sci. USA* **74** (12):5463-5467) by Genome Express S.A (Zone Astec, Grenoble, France) on both strands of the selected plasmids using T3, T7 universal primers and oligonucleotides derived from established sequence of each strand.

30

#### Cloning of the 5' end of the *BCVIR* cDNA

In order to obtain the full-length *BCVIR* cDNA sequence, the missing 5' end was obtained as follows: the cDNA library was used as a DNA template for a PCR experiment using the T3 sense universal primer derived from the vector sequence

(located 70 bp upstream of the *Eco*RI cloning site) and an antisense primer P2 (5'-ATGAGTCTATTGACTCCTTG-3') derived from the *BCVIR* cDNA sequence (nucleotide position 476-496 downstream of the *Eco*RI cloning site in the complete sequence of the cDNA, see Fig. 1 and Fig.2). The PCR was performed as described  
5 below (§ PCR amplification) using 1 µl of the cDNA library ( $10^5$  phages) as DNA template. The highest resulting PCR fragment (with a size of around 550 bp) was gel-extracted using gel extraction spin columns (Genomed), cloned in pGEM®-T vector using the pGEM®-T vector System II kit under the recommendations of Promega and sequenced on both strands as described behind, using T7 and SP6 universal primers  
10 (Genome Express).

#### **PCR amplification**

Amplifications were performed in a PTC-100™ Programmable Thermal Controller (MJ Research, INC.) as described in Carret *et al.* (1999, *J. Eukaryot. Microbiol.*,  
15 **46(3)**, 298-303) with the following conditions : 3 min. at 94°C, 30 cycles of 1 min. at 94°C, 1 min. at 55°C and 1 min. at 72°C, and then 5 min. at 72°C. Primers P1 (sense 5'-GACGTTGATGTGATGAGGAAGC-3'), P5 (sense 5'-AGGGAGCTGTCACGGAAGATT-3'), P2 (antisense) and P15.2 (antisense 5'-AATGACATACTCACAGGAAGC-3') were all derived from the *BCVIR* cDNA  
20 sequence (their respective position is indicated on the Fig.1) and commonly used for the molecular analysis of this sequence.

#### **Genomic DNA analysis of the *BCVIR* cDNA sequence:**

Genomic DNA extraction from *Babesia canis* cultures and Southern blot experiments  
25 were performed using standard procedures described in Maniatis/Sambrook (Sambrook J. *et al.*, Molecular cloning: a laboratory manual. ISBN 0-87969-309-6). The probes were obtained by performing PCR with the combinations of primers P1/P15.2 or P1/P2 using the pBK-CMV plasmid carrying the *BCVIR* cDNA as DNA template and they were labelled using Nick Translation kit under the  
30 recommendations of the manufacturer (Boehringer Mannheim). These probes were also tested on chromosomes of *B. canis* separated in 0.7% agarose (ICN) by pulse field gel electrophoresis (PFGE) and transferred onto Nylon membranes.

As control, hybridisation experiments were performed with a probe derived from the Bcc12D3 cDNA of *B. canis* A on the same membranes that were hybridized with probes derived from the *BCVIR* cDNA sequence. Dehybridisation was performed as recommended by Amersham Pharmacia Biotech.

5

## **RNA analysis of the *BCVIR* cDNA sequence:**

Total RNA was extracted using the RNeasy<sup>®</sup> Total RNA Isolation System according to Promega and was used as starting material for RT/PCR and Northern experiments.

10

**RT-PCR.** RT-PCR was performed using the two-step protocol of the Enhanced Avian RT-PCR kit as described by the manufacturer (Sigma). Briefly, the first step (retrotranscription) was performed for 50 min. at 42°C using 250 pg of total RNA as template and 1 µM of specific cDNA primer (sense P1, sense P5 (5'-AGGGAGCTGTCACGGAAGATT-3') or antisense P15.2), and the second step was performed at an annealing temperature of 50°C as described above (§PCR amplification), using one fifth of the first step reaction as a template. As controls, the first step was also performed without eAMV-RT for each primer tested for DNA contamination, and the experiments were also performed with specific primers (sense Bcc12D3.1 and antisense Bcc12D3.2) derived from the Bcc12D3 cDNA of *B. canis* A.

**Northern Blotting.** For Northern blot experiments, total RNA was electrophoresed through gel containing formaldehyde and transferred to Nylon membrane as described in Maniatis/Sambrook (Sambrook J. et al., Molecular cloning: a laboratory manual. ISBN 0-87969-309-6). Hybridisation was performed with a probe derived from a P1/P2 PCR fragment amplified on the *BCVIR* cDNA sequence and labelled with digoxigenin (DIG)-dUTP according the DIG High Prime DNA labelling kit (Boehringer Mannheim). Total RNA samples depleted in mRNA were also used for hybridisation experiments and mRNA depletion was performed using PolyA Tract mRNA Isolation Systems® III kit (Promega).

**Results:**

**Isolation and sequence of the *BCVIR* cDNA.** Immunoscreening of a cDNA library of *B. canis* (isolate A), using the anti-GST60.1 antiserum directed against one

5 member of the Pf60 multigene family of *P. falciparum* (Carcy *et al.*, 1994, *Mol. Biochem. Parasitol.* **68**: 221-233) allowed to isolate and sequence the *BCVIR* cDNA.

The complete nucleotide sequence of *BCVIR* cDNA contained 1135 nucleotides (between *Eco*RI to *Xba*I cloning restriction sites) with a poly(A)<sub>22</sub> tail (Fig.1A and Fig.2). An interesting feature of this cDNA is that two overlapping open reading

10 frames (ORF) are predicted (Fig.1A) : ORF1 between nucleotide 75 (ATG, initiation codon for Methionine) and 500 (TAA, stop codon) and ORF2 between nucleotides 448 (ATT codon for Isoleucine) and 933 (TGA, stop codon). These two ORF are overlapped by 52 nucleotides (position 448-500) and are +1 shifted (Fig.1A).

15 ORF1 encodes a predicted polypeptide of 141 residues (designated Bcvir15) starting by a methionine with an expected molecular mass of 15.7 kDa and an iso-electric point of 10.9 (Fig. 2). ORF2 does not contain a consensus translation initiation sequence and started by Ile (Fig.1A). It encodes a predicted polypeptide of 133 residues with an expected molecular mass of 15.2 kDa. Due to the +1 shift between ORF1 and ORF2, these two ORFs present different amino acid sequences in the 20 overlapping region (Fig.1B). Sequence analysis of the overlap region of *BCVIR* cDNA suggested that such a +1 ribosomal frame shift in that region could produce an ORF1-ORF2 fusion protein of 285 residues (designated Bcvir32), resulting from the translation of a 859 nucleotides sequence (Fig.3). Bcvir32 would have predicted molecular mass of 32.3 kDa and an iso-electric point of 10.5.

25

**DNA analysis:** The presence of *BCVIR* cDNA sequence in the genome of *B. canis* (isolates A and B) was analysed by PCR and hybridisation experiments both on DNA (Southern blot) or chromosomes (PFGE) from these isolates.

30 **PCR experiments.** These were performed using 3 combinations of specific primers from the *BCVIR* cDNA sequence : P1/P2 and P5/P2 covering the ORF1 (Fig.4A and 4B, respectively), and P5/P15.2 covering the ORF2 and the overlapping region between these two ORF of the cDNA (Fig.4C). As controls, these combinations of primers allowed to amplify DNA fragment of around 360, 160 and 630 bp on the

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cDNA *BCVIR* carried by the pBK-CMV plasmid (Fig.4A-C, lanes 5) and were negative on genomic DNA prepared from uninfected dogs (Fig.4A-C, lanes 4). These combinations of primers were unable to amplify genomic DNA fragments when they were tested on genomic DNA from the isolates A and B of *B. canis* (Fig.4, lanes 1 and 5, 2, respectively) whereas, as control for the DNA preparations, specific primers derived from the Bcc12D3 cDNA of *B. canis* were able to specifically amplify the expected 1200 bp genomic DNA fragment on these preparations (Fig.4D, lane 1 and 2, respectively). Moreover, primers from the *BCVIR* cDNA sequence were negative in PCR on genomic DNA from the isolate F of *B. rossi* as were those derived from the Bcc12D3 cDNA (Fig.4, lanes 3).

**Hybridisation experiments.** hybridisation with probes synthesised using the PCR fragments P1/P2 and P1/P15.2 from the *BCVIR* cDNA sequence, were performed on genomic DNA of isolates A and B of *B. canis* and isolate F of *B. rossi*, either digested 15 by various restriction enzymes (Southern blot) or on chromosomes (separated by PFGE). Whatever the hybridisation experiment performed, none of these probes allowed to detect the *BCVIR* sequence on genomic DNA of *B. canis*, whereas following de-hybridisation of these probes from the membranes, a probe derived from the Bcc12D3 cDNA sequence was able to detect its sequence on genomic DNA of *B. canis* (data not shown).

**RNA analysis.** Analysis of the *BCVIR* cDNA sequence was performed by RT-PCR (Fig.5, A-C) and hybridisation experiments (Northern blot) (Fig.6) using total RNA extracted from the isolate A of *B. canis*.

**RT-PCR experiment.** For the RT-PCR analysis of *BCVIR* sequence, analysis of the Bcc12D3 cDNA sequence was used as control (Fig.5D and 5E). As expected for this control, the 1200 bp DNA fragment from Bcc12D3 was amplified by PCR with the combination of primers Bcc12D3.1/Bcc12D3.2 when the first retrotranscription step 30 was performed with antisense primer Bcc12D3.2 (Fig.5E, lane 5), but not when it was performed with the sense primer Bcc12D3.1 (Fig.5D, lane 4). For the analysis of *BCVIR* sequence, the expected 630 bp DNA fragment was amplified by PCR with the combination of primers P5/P15.2 PCR when the first step of retrotranscription was performed with the antisense primer P15.2 (Fig.5C, lane 3). However and

unexpectedly, this DNA fragment of 630 bp was also amplified by PCR with this P5/P15.2 combination of primers when the first step of retrotranscription was performed with the sense primer P5 (Fig.5B, lane 2). Identically, a unexpected 360 bp DNA fragment was amplified by PCR with the P1/P2 combination of primers when 5 the first step of retrotranscription was performed with the sense primer P1 (Fig.5A, lane 1). The same experiments performed by omitting the e-AMV Retrotranscriptase enzyme in the retrotranscription steps did not allow to amplify DNA fragment by PCR with these combination of primers (Fig.5, lanes 1'-5'), demonstrating that the 360 and 630 bp DNA fragment amplified using the sense primers P1 or P5 in the 10 retrotranscription step were not from genomic DNA origin, as previously observed (§ DNA analysis). Moreover, their sequencing and their hybridisation with a probe derived from the entire *BCVIR* cDNA sequence (Fig.5, II) certified their specificity.

**Northern blot experiment.** hybridisation performed onto total RNA extracted from 15 *B. canis* (isolate A) with a probe corresponding to the ORF1 sequence of *BCVIR* cDNA allowed to detect three different RNA bands : a strong one of around 1400 bases length and two faint ones at around 1200 and 2800 bases length (Fig.6, lane 2). When hybridisation was performed with the same amount of starting total RNA but depleted in mRNA, the 1200 bases band disappeared (Fig.6, lane 3), indicating that 20 this RNA band is a mRNA, in agreement with the size of the cDNA. It confirms also that the *BCVIR* cDNA sequence is carried by non messenger RNA (the 1400 and 2800 bases length bands), in agreement with the results obtained by RT-PCR when the retrotranscription step was performed with sense primer.  
No signal was found when hybridised with the *BCVIR* cDNA sequence onto total 25 RNA from *B. rossi* (Fig.6, lane 4) or the bovine species *B. divergens* (Fig.6, lane 1).

**Conclusion:** The molecular characterisation of the *BCVIR* cDNA sequence indicates that it is not from *B. canis* genomic DNA origin. It demonstrates that it is carried by a mRNA, but also by a non messenger RNA of 1.4 kb and 3 kb. It demonstrates also 30 that the sequence from this cDNA is specifically found in the European isolates of *B. canis*, not in *Babesia rossi*.

**Example 2: Biochemical characterisation of proteins encoded by the BCVIR cDNA sequence.**

**Expression and purification of GST-ORF1 and GST-ORF2 recombinant**

5 **proteins in *E. coli*.**

In order to express the products encoded by ORF1 and ORF2 from the *BCVIR* cDNA as recombinant proteins, and to produce specific antisera against them, the two ORFs were subcloned in the *EcoRI/XhoI* cut dephosphorylated pGEX-4T3 vector (Amersham Pharmacia Biotech) in frame with the glutathione-S-transferase (GST).

10 In order to produce Bcvir15 protein encoded by ORF1 (aa M<sup>1</sup>-I<sup>141</sup>, Fig.2), the *EcoRI/XhoI* fragment containing the 1041 bp cDNA sequence (missing the 5' end of *BCVIR* cDNA) was excised from the pBK-CMV recombinant plasmid and subcloned into the vector. In order to obtain an antiserum specifically directed against the amino acid sequence deduced from the ORF2 sequence (aa sequence N<sup>154</sup>-C<sup>285</sup>, Fig.3), i.e.

15 excluding the 52 bp overlapping region between the two ORF (nucleotide position 448-500 in Fig.1), an *EcoRI* restriction site was created in the ORF2 sequence by a PCR experiment. Thus, the 1041 bp *BCVIR* cDNA sequence carried by the pBK-CMV plasmid was used as a template DNA for the PCR experiment with the sense primer P<sub>Eco</sub> (5'-ATGAGGAATTCTGAACCGACTA-3'; located at nucleotide position

20 529-549 on the complete *BCVIR* cDNA sequence, Fig.1 and Fig.2) and the antisense T7 universal primer derived from the vector sequence (located 75 bp downstream of the *XhoI* cloning site). The amplified fragment was cloned in pGEM®-T vector using the pGEM®-T vector System II kit under the recommendations of Promega. An *EcoRI/XhoI* fragment containing an around 600 bp DNA from ORF2 was released

25 from the recombinant plasmid and then subcloned into the vector. For both cloning, transformation with the ligation mixtures were performed into the *E. coli* BL21 cells and positive clones were selected using PCR with the specific primers from the *BCVIR* cDNA sequence.

30 Expression and purification of the recombinant GST-Bcvir15 protein and of a non recombinant GST from the pGEX-4T3 vector was performed according Smith and Johnson (1988, *Gene*, 67(1):31-40). However, the GST-ORF2 was unpurifiable using this standard procedure, because it aggregates in inclusion bodies. Thus, inclusion bodies were firstly isolated from total cell extract according the protocol of Nagai &

Thogersen (1987, In *Methods in enzymology* (ed. R. Wu and L. Grossman), 153, 461-481). They were then dissociated and loaded on a 12 % SDS-PAGE (Laemmli UK, 1974, *Nature (London)*, 287:680-685). Lastly, the recombinant GST-ORF2 was electro-eluted from the gel and was used to immunise a rabbit as described below.

5

**Production of antibodies directed against GST-ORF1 and GST-ORF2 proteins.**

**Polyclonal antisera.** Polyclonal antiserum raised against GST-ORF1 was produced both in mice and in a rabbit, and a polyclonal antiserum raised against GST-ORF2 was produced in a rabbit according E. Harlow & D. Lane (*Antibodies: a laboratory manual*. ISBN 2-907516-15-9). Rabbits (New Zealand White) were immunised with 50 µg of purified GST recombinant proteins emulsified in Freund's Complete

Adjuvant (FCA) (Sigma) subcutaneously for the first injection, and by intramuscular injections for the two subsequent ones using Freund's Incomplete Adjuvant (FIA) (Sigma). Balb/C mice were intraperitoneally immunised with 15 µg of GST-ORF1

emulsified in FCA for the first injection and in FIA for the two subsequent ones.

Injection of the animals was performed at 3-week intervals and the bleedings were done 8 days before each immunisation.

**Production and screening of monoclonal antibodies.** 48 h prior to fusion of

20 splenocytes from the mice immunised with the recombinant GST-ORF1 with X-63 Ag8-653 myeloma cells, a last injection of 15 µg of GST-ORF1 was performed. The fusion process and cloning by limiting dilution for the isolation of monoclonal antibodies were performed according to Brown et al. (1979, *J. immunol. Methods*, 31:201-209). Hybridoma supernatants were screened by indirect immunofluorescence

25 assay as described below.

**Immunological methods:**

**Immunoblotting.** Immunoblotting was performed with purified *B. canis* merozoites prepared as follows: parasitized erythrocytes from *in vitro* culture (20% of

30 parasitaemia) were extensively washed with sterile RPMI 1640 medium (Life Technologies). The pellet was lysed with α-hemolysin from *S. aureus* (Sigma) at a final concentration of 36U/ml during 30 min. at 37°C. Free merozoites and remaining unlysed cells were pelleted by centrifugation (3500 g, 30 min.) and the supernantant

was discarded. The pellet was washed one time with TBS, resuspended in 2 ml of TBS and the merozoites were purified by centrifugation (3500 g, 30 min.) on a gradient of saccharose formed by a lower layer of 1.09 density and a upper layer of 1.02 density. Free merozoites were harvested at the interface, they were pelleted by 5 centrifugation (15000 g, 10 min.) and washed 3 times with TBS. They were then processed for electrophoresis and the proteins were separated on a 15% SDS-PAGE. Immunoblotting was performed as described for *B. divergens* in Carcy *et al.* (1991, *Biol. Cell*, 72, 93-102) using a 1:100 dilution for the polyclonal antisera directed against GST-ORF1 and GST-ORF2 proteins.

10

**Indirect immunofluorescence assays (IFA).** IFA and double IFA were performed on *B. canis* parasitized erythrocytes (5% of parasitaemia) as described for *P. falciparum* in Grellier *et al.* (1994, *Biol. Cell*, 82, 129-138) using a 1:200 dilution for the polyclonal antisera directed against GST-ORF1 and GST-ORF2 proteins. For double 15 IFA, the slides were first incubated with the anti-GST-ORF2 rabbit antiserum and then with mouse polyclonal or monoclonal antibodies directed against the GST-ORF1. Slides were mounted with Citifluor solution (Citifluor Ltd, London, UK) for limiting extinction fluorescence and the fluorescence was detected using a fluorescence microscope (Axioscope, Zeiss).

20

**[<sup>35</sup>S]-methionine radiolabelling of *B. canis* culture and immunoprecipitation.** [<sup>35</sup>S]-methionine radiolabelling of *in vitro* cultures of *B. canis* with 50 µCi/ml (1200 Ci/mmol, Amersham-Pharmacia Biotech) and a 5% starting parasitaemia, and immunoprecipitation assays were performed as described for *B. divergens* in Carcy *et al.* (1991, *Biol. Cell*, 72, 93-102).

**Protein phase separation by TX-114 treatment.** The proteins of *B. canis* were phase separated in Triton X-114 (Sigma) as described for *B. divergens* in Precigout *et al.* (1991, *Infect. Immun.*, 59(8), 2799-2805) using 200 µl of [<sup>35</sup>S]-methionine 30 radiolabeled parasitized red blood cells (20% of parasitaemia) as starting material.

**In Vitro translation of the BCVIR cDNA sequence.** The ability of the BCVIR cDNA to direct frame shifting was examined in an *in vitro*-translation system. *In vitro*-

translation products were synthesised using the TNT® Quick Coupled Transcription/Translation System (Promega) with a PCR DNA fragment containing the entire *BCVIR* cDNA sequence, a Kozacks sequence and a T7 promoting sequence at its 5' end for the *in vitro* translation. In order to amplify such a DNA fragment, the

5 PCR was performed on the pBK-CMV plasmid carrying the incomplete 1041 bp *BCVIR* cDNA as DNA template using the two following primers: a 90 mer sense oligonucleotide (primer P0 : 5'-  
 GGATCCTAATACGACTCACTATAAGGGAGACCACCATGGAGTCGACATCAA  
 CAG ACCAACTTGTGCCGAGAACCGTCCCACCTTG-3'), containing a  
 10 Kozacks and a T7 promoting sequences but also the missing 5' end of the entire *BCVIR* cDNA sequence containing the starting ATG codon of the ORF1 (bolded in the primer sequence; nucleotide position 75-130 on the complete cDNA sequence; see Fig.1 and Fig.2), and the antisense primer P15.2 from the *BCVIR* cDNA sequence.  
 The PCR was performed using 100 ng of the circular recombinant pBK-CMV plasmid  
 15 and the following conditions of amplification were used: 1 cycle of 3 min. at 94°C; 5 cycles of 1 min. at 94°C, 1 min. at 37°C and 1 min. at 72°C; 25 cycles of 1 min. at 94°C, 1 min. at 50°C and 1 min. at 72°C. The PCR products were gel extracted twice to ensure their purity and resuspended in nuclease free water at a concentration of 100 ng/μl. Then, they were added into a reticulocyte lysate to be translated using the  
 20 TNT® Quick Coupled Transcription/Translation System as described by the manufacturer (Promega). As control, the same experiment was performed by omitting the PCR fragment in the reaction. The total radiolabeled translated products (5 μl of the reaction) and immunoprecipitated products (from 20 μl of the reaction) with the anti-GST-ORF1 or the anti-GST-ORF2 or the anti-GST antisera and the pre-immune  
 25 rabbit serum were separated on a 15% SDS-PAGE.

## Results:

30 **Purification of GST-ORF1 and GST-ORF2.** In order to produce polyclonal antibodies specifically directed against polypeptide encoded by ORF1 or ORF2, the 141 residues ( $^1\text{M}$ - $^{141}\text{I}$ ) of ORF1 (encoded from  $^{75}\text{ATG}$  to the stop codon  $\text{TAA}^{500}$ ) (Fig.2) and 132 residues ( $^{154}\text{N}$ - $^{285}\text{C}$ ) of the ORF2 (encoded from nucleotide position

<sup>533</sup>ATT to the stop codon TGA<sup>933</sup>) (Fig.3) were cloned in the pGEX-4T3 vector in frame with the GST sequence. Overexpression of the GST-ORF1 and GST-ORF2 were performed in the BL21 *E. coli* strain (Fig. 7). Expression studies demonstrated that *E. coli* cultures containing the recombinant pGEX 4T3-ORF1 expressed, after

5 IPTG induction, the GST-ORF1 protein as a soluble protein in the lysate of sonicated bacteria (Fig. 7A, lane 1). After elution, the soluble GST-ORF1 was purified and it presented the expected molecular mass of 40 kDa (Fig. 7A, lane 2). In contrast, the GST-ORF2 was insoluble in *E. coli* cultures containing the recombinant pGEX 4T3-ORF2 and the recombinant protein, with a molecular mass of 39 kDa, was mainly found in inclusion bodies (Fig. 7B, lane 5). In order to immunise a rabbit with the purified GST-ORF2, it was gel excised from an extract of purified inclusions bodies separated on 12% SDS/PAGE (Fig. 7B, lane 4).

10

As control, a GST was purified after IPTG induction of an *E. coli* culture containing the native pGEX 4T3 (Fig. 7B, lane 3).

15

**Immunological studies:** Polyclonal antisera, raised against GST-ORF1 or GST-ORF2 or GST, were produced by immunising mice and rabbits in order to perform immunological studies of the cDNA encoded polypeptides. Two monoclonal antibodies directed against GST-ORF1 were also tested.

20

**Immunoprecipitation experiments.** in a first experiment, they were performed both on the total (Fig.8A) and exoantigens (Fig.8B) radiolabelled fractions of *B. canis* cultures (isolate A). In the total antigen fraction, the anti-GST-ORF1 strongly recognises a major product of 15 kDa (Bcvir15, indicated by an asterisk) and a minor product of around 32 kDa (putative Bcvir32, indicated by an arrow) (Fig.8A, lane 3). In the exoantigen fraction, only the minor product of around 32 kDa was detected faintly (Fig.8B, lane 3). The anti-GST-ORF2 was found not reactive against a 32 kD protein on the total or exoantigen fractions (Fig.8, lanes 4). Pre-immune rabbit and anti-GST antisera were not reactive on the antigens recognised with the anti-GST-ORF1 antiserum (Fig.8, lanes 1 and 2, respectively). In a second experiment, the hydrophylic and hydrophobic fractions of *B. canis* (isolate A) antigens, obtained following TX-114 treatment, were analysed by immunoprecipitation with the anti-GST-ORF1 (Fig.9). It allowed to detect the 15 kDa protein is the hydrophilic fraction (Fig.9A, lane 2, indicated by an asterisk) and no reactivity was observed in the

hydrophobic fraction (Fig.9B, lane 2). As control, no reactivity was observed against the 15 kDa protein when the immunoprecipitation was performed with the pre-immune rabbit serum (Fig.9, lanes 1). MAb directed against GST-ORF1 were not reactive in immunoprecipitation experiments.

5

**Western blot.** Western blots were performed with purified merozoites from the isolate A of *B. canis*. The anti-GST-ORF1 specifically recognised the 15 kDa protein (Fig.10A, lane 2, indicated by an asterisk) but, in contrast to immunoprecipitation experiments, it did not recognise the minor 32 kDa product. However, the anti-GST-10 ORF2, which recognised different proteins in Western blot experiment, was able to detect such a 32 kDa antigen (Fig.10B, lane 2, indicated by an arrow). No protein of around 15 kDa (which could correspond to predicted 15.2 kDa polypeptide of the ORF2) was recognised by the anti-GST-ORF2. As controls, pre-immune rabbit serum from each antiserum were not reactive in western blot (Fig.10, lanes 1). MAb directed 15 against GST-ORF1 were not reactive in Western blot.

**Immunofluorescence assays.** IFA tests were performed on fixed blood smears from *in vitro* cultures of the isolates A of *B. canis* (Fig.11) with the anti-GST-ORF1 and the anti-GST-ORF2. The fluorescence obtained with the polyclonal rabbit anti-GST-

20 ORF1 was associated with numerous intraparasitic vesicles, dispersed through all the parasite cytoplasm but excluding the parasite's nucleus (Fig.11, picture A). The same pattern of immunofluorescence was observed with the mAb directed against GST-ORF1. Moreover, a double IFA performed with the mAb directed against GST-ORF1 and the polyclonal rabbit anti-GST-ORF2 indicated that antigens recognised by anti-GST-25 ORF2 (Fig.11, picture B) co-localised with those recognised by the anti-GST-ORF1. As controls, anti-GST and pre-immune rabbit antiserum were not reactive in IFA tests. The same profile of fluorescence was observed when the IFA tests were performed on infected erythrocytes from the isolate B of *B. canis* (data not shown).

30 ***In vitro* translation of the BCVIR cDNA.** An *in vitro* translation of the entire BCVIR cDNA sequence was performed in reticulocyte lysate in order to analyse, by immunoprecipitation experiments, its products of expression (Fig.12, lanes 3-4). Analysis of total expression products revealed that a major 15 kDa protein was expressed when the experiment was performed with the cDNA (Fig.12, lane 4;

indicated by an asterisk) but not when it was performed by omitting the cDNA as control (Fig.12, lane 1). This major 15 kDa protein was specifically immunoprecipitated by the anti-GST-ORF1 but also a minor protein of around 32 kDa (Fig.12, lane 3; indicated by an arrow). As controls, the pre-immune rabbit serum and 5 the anti-GST antiserum were not reactive on these products (data not shown). Thus, it suggests that such a minor 32 kDa product could result from the predicted +1 frame shift mechanism. As was the case for immunoprecipitation assays previously described, the anti-GST-ORF2 was not reactive on this minor product (data not shown).

10 **Conclusion:** The anti-GST-ORF1 antiserum is able to detect two proteins, a hydrophilic major one of 15 kDa and a minor one of 32 kDa, the last one being detected in the supernatant of *in vitro* culture of *B. canis*. The anti-GST-ORF2 antiserum was also able to detect a 32 kDa protein, but was unable to detect a 15 kDa 15 product. It indicates that the ORF1 of the *BCVIR* cDNA encodes for a 15 kDa protein (predicted 15.7 kDa product, designated Bcvir15) and that ORF2 is unable to synthesise the predicted 15.2 kDa product. Moreover, the detection of a 32 kDa protein commonly recognised by anti-GST-ORF1 and anti-GST-ORF2 antisera, suggest that it could correspond to the predicted 32.3 kDa product resulting from a +1 20 frame shift mechanism and allowing to produce a fused ORF1-ORF2 protein (designated Bcvir32). The *in vitro* translation of the *BCVIR* cDNA sequence indicated that such a mechanism might occur in the cDNA sequence. The low level of expression of a 32 kDa protein in comparison with the high expression level of Bcvir15 protein is in agreement with such a mechanism. Moreover, the co-localisation 25 of proteins recognised by the two antisera in intraparasitic vesicles, indicating that the 32 kDa protein recognised by the two antisera is the same one, is consistent with such a frame-shift mechanism.

30 **Example 3- *Babesia canis* growth-inhibition tests.**

**Inhibition test by antisera**

Before each experiment, the parasitaemia was adjusted to 1% with a 2% haematocrit (day D0). *B. canis* parasites (in conditions previously described in § growth of *B.*

*canis*) were *in vitro* cultured in 1 ml for experiments performed in 24 wells plates or in 2 ml for experiments performed in 6 wells plates. The ability of the anti-GST-ORF1 and anti-GST-ORF2 antisera to inhibit *B. canis* growth *in vitro* was determined by adding each antiserum to the medium at different concentrations (from 1 to 8%).

5 Controls were : i) incubation with the rabbit pre-immune serum or the rabbit anti-GST serum or with the irrelevant rabbit anti-GST-Bcc12D3 serum at same concentrations; ii) a *B. canis* *in vitro* culture without treatment.

The ability of each concentration of antiserum to inhibit *in vitro* growth of *B. canis* (isolate A and B), was performed in triplicate and determined by parasitaemia on

10 Giemsa-stained smears or by metabolic labelling with 50 µCi/ml of [<sup>3</sup>H]-Hypoxanthine in aqueous solution (ICN), after increasing incubation times of 24 h (day D1) and 48 h (day D2) corresponding respectively to about 3 and 6 *B. canis* life cycles. After D1, half of the volume of the wells was collected and the culture medium was entirely removed and replaced by fresh medium supplemented with  
 15 antiserum (and [<sup>3</sup>H]-Hypoxanthine for labelling experiment) at the same concentration used at D0. The collected infected erythrocytes (respectively 5 and 20µl in 24 and 6 wells plates) were pelleted by centrifugation at 1800 g for 5 min. and washed five times in TBS. For the determination of radioactivity incorporation, they were lysed in 500µl of RIPA buffer and placed 1 h on ice. The lysates were  
 20 centrifuged at 15000 g (15 min., 4 °C), 2 µl of the supernatant were then added to 2 ml of Emulsifier-Safe (Packard, USA) and the incorporated radioactivity was measured.

#### **In vitro inhibition assay with purified immunoglobulins.**

25 Before each experiment, the parasitaemia was adjusted to 1% with a 2% haematocrit (day D0). *B. canis* and *B. rossi* parasites were cultured *in vitro* in 96 wells plates in 100 µl of medium . Antisera used for inhibition assays (anti-GST-Bcvir15, anti-GST-Bcvir15 pre-immune, anti-GST-Bcc12D3, anti-GST-ORF2 antiserum and anti-GST (Respectively indicated as Bcvir15, T0, CONT, orf2 and GST in fig. 17)) were first  
 30 purified by FPLC on a HITrap™ Protein G column (Amersham Pharmacia Biotech). Purified immunoglobulins were then dialyzed overnight at 4°C versus PBS 1X buffer and total immunoglobulins were titrated using the Coomassie Protein Reagent Kit (Pierce, USA). ELISA was used to evaluate the ratio of immunoglobulins directed either against a native GST protein or against each recombinant protein fused to GST

by comparison of reactivity of purified immunoglobulins on 0.1 µg of non recombinant GST or 0.1 µg of recombinant proteins coated in wells. All purified immunoglobulin samples were then adjusted at the same concentration of specific anti-GST immunoglobulins with RPMI. The ability of each sample to inhibit *in vitro* culture of *Babesia canis* or *Babesia rossi* was performed in triplicate with 1, 2, 4, 8 and 10 µg of specific immunoglobulins directed against the non GST part of the recombinant protein used to obtain the antisera. The growth inhibition was evaluated by metabolic labelling with 50 µCi/ml of [<sup>3</sup>H]-Hypoxanthine in aqueous solution (ICN), during 24 hours (about 3 cycles of *B. canis* life cycle). Then, the erythrocytes were blocked on a filter and washed using a Scatron apparatus. Afterward, the radioactivity associated with each well was read in 2 ml of Scintillant (Emulsifier Safe™ Packard, USA) by scintigraphy.

#### **Reversion of the inhibition by recombinant GST-ORF1 protein**

The ability of the recombinant GST-ORF1 to reverse the inhibitory effect of the anti-GST-ORF1 on growth of *B. canis* was tested on *in vitro* culture maintained as described above. Different concentrations of recombinant protein in the medium containing 8% of anti-GST-ORF1 were tested: 20 µg/ml and 40 µg/ml. Four controls were used: i) *B. canis* *in vitro* culture without treatment; ii) culture containing 20 µg/ml or 40 µg/ml of recombinant GST-ORF1; iii) a culture containing 8% of anti-GST-ORF1 antiserum.

The ability of each concentration of GST-ORF1 to reverse the inhibitory effect of the anti-GST-ORF1 antiserum was performed in triplicate and determined by parasitaemia on Giemsa-stained smears at D0, D1 and D2 as described above.

25

#### **Results:**

**Inhibition test by antisera.** In a first experiment, the ability of the different bleedings from the anti-GST-ORF1 antiserum (noted II, III and IV in the figure 13) to inhibit the *in vitro* growth of *B. canis* (isolate A) was tested by adding in the culture medium different concentration of each bleeding (from 1 to 8%, respectively noted A to D in the figure 13). Compared to the control culture, where parasitaemia increased from 1 to 35% during the 2 days experiment (Fig.13E), a significant inhibitory effect of the anti-GST-ORF1 on the *in vitro* growth of *B. canis* was observed at D2 with the

second and the third bleedings of the antiserum at a concentration of 8% since the parasitaemia was of around 15% at D2 in these treated cultures (Fig.13D, III and IV, respectively). As control, no inhibitory effect of the pre-immune rabbit serum was observed whatever the concentration used (Fig.13, I).

5 Another experiment was performed in order to analyse the inhibitory effect of the anti-GST-ORF2 antiserum on the growth of *B. canis* (Fig.14). It was tested and compared to anti-GST-ORF1 antiserum, and pre-immune or anti-GST or irrelevant anti-GST-Bcc12D3 rabbit antisera were used as controls (Fig.14). All antisera were tested at the inhibitory concentration of anti-GST-ORF1 (8%). In that experiment, a  
10 significant inhibitory effect of the anti-GST-ORF2 was also observed at D2 since the parasitaemia was of 22% in the treated culture whereas it was of 35% in the control culture. Moreover, the inhibitory effect of anti-GST-ORF2 was found lower than the one observed with the anti-GST-ORF1 (at D2 the parasitaemia is of around 15%). No inhibitory effect was observed by treating the culture with 8% of pre-immune or anti-  
15 GST or anti-GST-Bcc12D3 rabbit antisera.

The 8% inhibitory effect of the anti-GST-ORF1 antiserum was also checked by measuring incorporation of [<sup>3</sup>H]-Hypoxanthine following metabolic labelling of treated and untreated cultures, and the experiment was performed on the isolates A and B of *B. canis* (Fig.15, A and B respectively). For both isolates and compared to  
20 the control cultures, a significant inhibitory effect was observed at D2 since the radioactivity was of around 2000 cpm in the untreated cultures and respectively of 250 and 350 cpm in the isolate A and B. In contrast to parasitaemia counting which was unable to detect an inhibitory effect of the anti-GST-ORF1 at D1, this inhibitory effect was visible at this day by radioactivity counting. Indeed, radioactivity in the  
25 control culture was of 1000 cpm whereas in treated cultures it was of 200 and 250 cpm respectively in the isolate A and B.

***In vitro* inhibition assay with purified immunoglobulins.**

The experiment described above was repeated with purified immunoglobulins, in  
30 order to exclude non-immunoglobulin serum-components as a possible non-specific cause of growth inhibition. As is shown in figure 17, purified immunoglobulins are capable of inhibiting *in vitro* growth of both *Babesia canis* A and B.

Surprisingly, this implies that vaccines comprising one or both proteins according to the invention are capable of inhibiting parasitic growth of *B. canis* in general, regardless the serovar. As expected, *in vitro* growth of *B. rossi* M is not inhibited.

5 **Reversion of the inhibition.** In order to confirm the inhibitory effect of anti-GST-  
ORF1 on the *in vitro* growth of *B. canis*, an experiment was conducted to reverse this  
effect by adding in the culture different concentration of purified recombinant GST-  
ORF1 (Fig.16). The two concentrations used were 20 and 40 µg/ml of GST-ORF1.  
This experiment showed that addition of GST-ORF1 protein in the medium of a  
10 culture treated with 8% of anti-GST-ORF1 allowed a 50 and 100% reversion of the  
inhibition process respectively at concentration of 20 and 40 µg/ml (Fig.16, C and D  
respectively). Such a concentration of GST-ORF1 added in a non treated culture had  
no effect on the growth of the parasite (Fig.16, A and B respectively). Untreated  
culture and a treated culture with 8% of anti-GST-ORF1 were used as controls for this  
15 experiment (Fig.16, E and F, respectively).

**Conclusion:** Both anti-GST-ORF1 and anti-GST-ORF2, at a concentration of 8% were able to inhibit, significant and specifically, the *in vitro* growth of *B. canis* isolate. This effect was already observed at D1. A concentration of 40 µg/ml of recombinant GST-ORF1 was able to totally reverse this inhibitory effect. Moreover, filtration of anti-GST-ORF1 and anti-GST antisera indicated that 8% of each antiserum represents a respective concentration of immunoglobulins of 100 and 50 µg/ml of culture (data not shown). An ELISA test, performed on coated recombinant GST, indicated that half of the immunoglobulins from the anti-GST-ORF1 were directed against the GST part of the recombinant GST-ORF1 (data not shown), it means that the inhibitory effect observed at 8% of anti-GST-ORF1 on the *in vitro* growth of *B. canis* was obtained by 50 µg/ml of immunoglobulins specifically directed against the protein Bcvir15. At least, this is consistent with the 40 µg of recombinant GST-ORF1 necessary to reverse the inhibitory effect of the antiserum anti-GST-ORF1.

Thus, these data indicated that Bcvir15 and Bcvir32 (products of the *BCVIR* cDNA), for which the genetic information is present in *B. canis* but not encoded by the *B. canis* genomic DNA play an important role in the virulence of the Apicomplexan

parasite *B. canis*. Vaccines based upon these proteins or nucleic acid sequences encoding these proteins are shown to play an important role in the inhibition of *B. canis* parasite growth, regardless the serovar.

**Figure 1: Schematic representation of the *BCVIR* cDNA**

A: Schematic representation of entire *BCVIR* cDNA with predicted ORFs represented by boxes and sequence derived primers represented with arrows

5      B: Representation of the predicted overlapping sequence and its deduced amino acid sequence

**Figure 2: Nucleic sequence of the complete *BCVIR* cDNA and deduced amino acid sequence of the Bevir15.**

10     The common amino acid sequence between Bcvir15 and the putative Bcvir32 is represented in regular upper case. A GRAM-positive anchoring hexapeptide is overlined. The overlapping sequence between the two predicted ORF of *BCVIR* cDNA are indicated in bold italic. Cloning restriction sites are in **bold case**.

15     **Figure 3: Nucleic sequence of the complete *BCVIR* cDNA and deduced amino acid sequences of the putative Bcvir32 protein.**  
The overlapping region between ORF1 and ORF2 of the *BCVIR* cDNA is indicated in ***bold italic***. Cloning restriction sites are in **bold case**.

20     **Figure 4: PCR analysis of *BCVIR* cDNA sequence on genomic DNA of *B. canis***  
Genomic DNA analysed were extracted from isolate A of *B. canis* (lane 1), isolate B of *B. canis* (lane 2), isolate F of *B. rossi* (lane 3) and uninfected dog blood sample (lane 4). The pBK-CMV plasmid carrying the *BCVIR* cDNA sequence was used as positive control (lane 5). PCR were performed using the combination of primers

25     P1/P2 (A), P5/P2 (B), P5/P15.2 (C) and Bcc12D3.1/Bcc12D3.2 (D)

**Figure 5: RT-PCR analysis of the *BCVIR* sequence**

The experiment was performed on total RNA isolated from *B. canis* (isolate A). P1 (lane 1), P5 (lane 2), P15.2 (lane 3); all derived from *BCVIR* sequence and Bcc12D3.1 (lane 4) or Bcc12D3.2 (lane 5) of *Bcc12D3* sequence were used as initial primers for the retrotranscription step. The first step of retrotranscription was performed either with eAMV-RT enzyme (lane 1-5) or without eAMV-RT enzyme as control (lane 1' - 5'). The second step of amplification was performed by PCR with the combination of primers P1/P2 (A), P5/P15.2 (B, C) and Bcc12D3.1/Bcc12D3.2 (D). Amplified

products were separated on 0.8% agarose gel and visualised by Bet staining under UV light (I) or they were transferred on to Nitrocellulose membrane and hybridised with a total *BCVIR* cDNA sequence used as a probe in order to check the specificity of the amplified PCR products (II).

5

**Figure 6:**Northern blot analysis of the *BCVIR* cDNA sequence

Hybridisation was performed using the PCR derived P1/P2 probe of *BCVIR* cDNA sequence on total RNA from the isolate Rouen 1987 of *B. divergens* (lane 1), total RNA from the isolate A of *B. canis* (lane 2), non messenger RNA from the isolate A of *B. canis* (lane 3) and total RNA the isolate F of *B. rossi* (lane 4).

10 of *B. canis* (lane 3) and total RNA the isolate F of *B. rossi* (lane 4).

**Figure 7: Over expression and purification of the GST-ORF1 and GST-ORF2 recombinant proteins from *E. coli***

Proteins were purified from BL21 (DE3) cells after 3 h IPTG induction as described,

15 separated on a SDS/12% polyacrylamide gel, and stained with Coomassie brilliant blue.

Lanes : 1A, *E. coli* expressing GST-ORF1 lysate ; 1B, purified GST-ORF1; 3B control purified native GST; 4B purified GST-ORF2; inclusion bodies lysate from *E. coli* expressing GST-ORF2.

20

**Figure 8: Immunoprecipitation of [<sup>35</sup>S] methionine labelled *Babesia canis* isolate A antigens**

Radiolabelled Total antigens (A) or Exoantigens (B) were immunoprecipitated respectively by rabbit pre-immune sera (1), anti-GST rabbit polyclonal serum (2), anti-GST-ORF1 rabbit polyclonal serum (3) and anti-GST-ORF2 rabbit polyclonal serum. Arrows and asterisk indicate respectively the location of the Bcvir15 and the putative Bcvir32.

**Figure 9: Immunoprecipitation of [ $^{35}$ S] methionine labelled *Babesia canis* A strain TX-114 partitioned antigens**

6

The [ $^{35}\text{S}$ ] methionine labelled *in vitro* cultures of *Babesia canis* A isolate were solubilized in TX-114, and after a phase separation, the hydrophilic were recovered in the aqueous phase and the amphiphilic antigens were recovered in the detergent phase. Pre-immune rabbit serum (1) or anti-GST-ORF1 rabbit polyclonal serum (2)

were used to immunoprecipitated either the aqueous-phase proteins (A) or the detergent phase proteins (B). Arrow indicate the location of the Bcvir15.

**Figure 10: Immunoblot analysis of SDS-PAGE separated *Babesia canis***

5 **merozoite antigens**

The experiment was performed on purified merozoites of the isolate A of *Babesia canis*. Antigens were separated on a 12% SDS/polyacrylamide gel and transferred onto nitrocellulose sheets. Incubation was done at a 1 : 100 dilution with pre-immune rabbit serum (1A and 1B), with anti-GST-ORF1 rabbit polyclonal serum (2A) or with 10 anti-GST-ORF2 rabbit polyclonal serum (2B). The position of the Bcvir15 and Bcvir32 are indicated respectively by an asterisk and an arrow.

**Figure 11: Localisation of BCVIR sequence expressed products.**

IFA tests were performed with the isolate A of *Babesia canis*.

15 (A) fluorescence associated with anti-GST-ORF1  
 (B) fluorescence associated with anti-GST-ORF2

**Figure 12: Analysis of *in vitro* translated products.**

Radiolabelled *In vitro* products translated from P0/P15.2 PCR amplification on pBK-  
 20 CMV BCVIR cDNA were separated on a 12% SDS-PAGE. Total products (4) or products immunoprecipitated by anti-GST-ORF1 (3) were analysed. Total expression product of reticulocyte lysate without DNA (1) or immunoprecipitated product from a reticulocyte lysate without DNA (2) were used as controls. Asterisk localise Bcvir15 and arrow localise Bcvir32.

25

**Figure 13: evaluation of the capacity of different concentration of anti-GST-ORF1 rabbit polyclonal sera to inhibit *Babesia canis* A strain *in vitro* culture.**

Different bleeding (respectively first (II), second (III) and third (IV) anti-GST-ORF1 rabbit polyclonal serum were added at a final concentration of 1% (A), 2% (B), 4% 30 (C) and 8% (D) to *Babesia canis* A *in vitro* culture. Pre-immune serum (I) was used as control as was culture maintained in standard conditions (E)

**Figure 14: Inhibition of *Babesia canis* A strain *In vitro* culture by different rabbit polyclonal sera**

Sera were tested for their ability to inhibit parasite growth when added at a rate of 8% on *Babesia canis* A strain *in vitro* culture. D0, D1, D2 indicate respectively parasitaemia values at the start of the test, after 24 hours of culture growth and after 48 hours of culture growth. Tested sera were rabbit polyclonal anti-GST-ORF1 and 5 anti-GST-ORF2 and control sera were rabbit pre-immune serum, rabbit polyclonal anti-GST and rabbit polyclonal anti-GST-Bcc12D3. Control lane indicate culture in standard conditions without addition of any rabbit serum.

**Figure 15: Inhibition test of *Babesia canis* *in vitro* culture evaluated by [<sup>3</sup>H] hypoxanthine metabolic labelling**

Anti-GST-ORF1 and control pre-immune rabbit sera were all tested at 8% final concentration on *Babesia canis* A strain (I) and *Babesia canis* B strain (II). Control lane indicate in both case parasites grown in standard conditions.

**Figure 16: Inhibition test against GST-ORF1 recombinant protein**

The recombinant GST-ORF1 protein was tested for its ability to revert the capacity of rabbit polyclonal anti-GST-ORF1 serum to inhibit *Babesia canis* A strain growth *in vitro*. The protein was tested at 20µg/ml (C) or 40µg/ml (D) against 8 % of anti-GST-ORF1 rabbit polyclonal serum. Controls were protein alone added in the culture at a 20 rate of 20µg/ml (A) or 40µg/ml (B), culture in standard conditions (E) and culture with 8% of anti-GST-ORF1 rabbit polyclonal serum (F)

**Figure 17: Inhibition test of *Babesia canis* *in vitro* culture with purified immunoglobulins evaluated by [<sup>3</sup>H] hypoxanthine metabolic labelling.**

25 The ability of purified immunoglobulins (anti-GST-Bcvir15, anti-GST-Bcvir15 pre-immune, anti-GST-Bcc12D3, anti-GST-ORF2 antiserum and anti-GST (Respectively indicated as Bcvir15, T0, CONT, orf2 and GST in fig. 17) to inhibit *in vitro* culture of *Babesia canis* or *Babesia rossi* was performed in triplicate with 1, 2, 4, 8 and 10 µg of specific immunoglobulins. Growth inhibition was evaluated by metabolic labelling 30 with 50 µCi/ml of [<sup>3</sup>H]-Hypoxanthine.

## **Claims**

- 1) Nucleic acid sequence encoding a 15 kD *Babesia canis* associated protein or an immunogenic fragment of said protein, said protein or immunogenic fragment thereof having at least 80 %, preferably 90 %, more preferably 95 % homology with the amino acid sequence as depicted in SEQ ID NO: 2.
- 2) Nucleic acid sequence encoding a 32 kD *Babesia canis* associated protein or an immunogenic fragment of said protein, said protein or immunogenic fragment thereof having at least 80 %, preferably 90 %, more preferably 95 % homology with the amino acid sequence as depicted in SEQ ID NO: 4.
- 3) cDNA fragment comprising a nucleic acid sequence according to claim 1 or 2.
- 4) Recombinant DNA molecule comprising a nucleic acid sequence according to claim 1 or 2 or a cDNA fragment according to claim 3, under the control of a functionally linked promoter.
- 5) Live recombinant carrier comprising a cDNA fragment according to claim 3 or a recombinant DNA molecule according to claim 4.
- 6) Host cell comprising a nucleic acid sequence according to claim 1 or 2, a cDNA fragment according to claim 3, a recombinant DNA molecule according to claim 4 or a live recombinant carrier according to claim 5.
- 7) *Babesia canis* associated protein, said protein having a molecular weight of 15 kD and comprising an amino acid sequence that is at least 80 % homologous to the amino acid sequence as depicted in SEQ ID NO: 2 or an immunogenic fragment of said protein.
- 8) *Babesia canis* associated protein according to claim 7, wherein the amino acid sequence is at least 85 %, preferably 90 %, more preferably 95 % homologous to the amino acid sequence as depicted in SEQ ID NO: 2, or an immunogenic fragment of

said protein.

9) *Babesia canis* associated protein, said protein having a molecular weight of 32 kD and comprising an amino acid sequence that is at least 80 % homologous to the amino acid sequence as depicted in SEQ ID NO: 4 or an immunogenic fragment of said protein.

10) *Babesia canis* associated protein according to claim 9, wherein the amino acid sequence is at least 85 %, preferably 90 %, more preferably 95 % homologous to the amino acid sequence as depicted in SEQ ID NO: 4, or an immunogenic fragment of said protein.

11) *Babesia canis* associated protein according to claims 7-10 for use in a vaccine.

12) Use of a *Babesia canis* associated protein according to claims 7-10 for the manufacturing of a vaccine for combating *Babesia canis* infections.

13) Vaccine for combating *Babesia canis* infections, characterised in that it comprises a nucleic acid sequence according to claim 1 or 2, a cDNA fragment according to claim 3, a recombinant DNA molecule according to claim 4, a live recombinant carrier according to claim 5, a host cell according to claim 6 or a protein according to claims 7-10, and a pharmaceutically acceptable carrier.

14) Vaccine according to claim 13, characterised in that it comprises an adjuvant.

15) Vaccine according to claim 13 or 14, characterised in that it comprises an additional antigen derived from a virus or micro-organism pathogenic to dogs or genetic information encoding said antigen.

16) Vaccine according to claim 15, characterised in that said virus or micro-organism pathogenic to dogs is selected from the group of *Ehrlichia canis*, *Babesia gibsoni*, *vogeli*, *rossi*, *Leishmania donovani*-complex, Canine parvovirus, Canine distempervirus, *Leptospira interrogans* serovar *canicola*, *icterohaemorrhagiae*,

*pomona, grippotyphosa, bratislava, Canine hepatitisvirus, Canine parainfluenzavirus, rabies virus, Hepatozoon canis and Borrelia burgdorferi*

17) Vaccine for combating *Babesia canis* infections, characterised in that it comprises antibodies against a protein according to claims 7-10, or an immunogenic fragment thereof.

18) Method for the preparation of a vaccine according to claims 13-16, said method comprising the admixing of a nucleic acid sequence according to claim 1 or 2, a cDNA fragment according to claim 3, a recombinant DNA molecule according to claim 4, a live recombinant carrier according to claim 5, a host cell according to claim 6 or a protein according to claims 7-10 and a pharmaceutically acceptable carrier.

19) Method for the preparation of a vaccine according to claim 17, said method comprising the admixing of said antibodies and a pharmaceutically acceptable carrier.

20) Diagnostic test for the detection of *Babesia canis* associated RNA characterised in that the test comprises a nucleic acid sequence that is at least 70 % homologous to the nucleic acid sequence as depicted in SEQ ID NO: 1 or 3 or a nucleotide sequence that is complementary to said nucleic acid sequence, or a fragment thereof having a length of at least 12, preferably 15, more preferably 18 nucleotides.

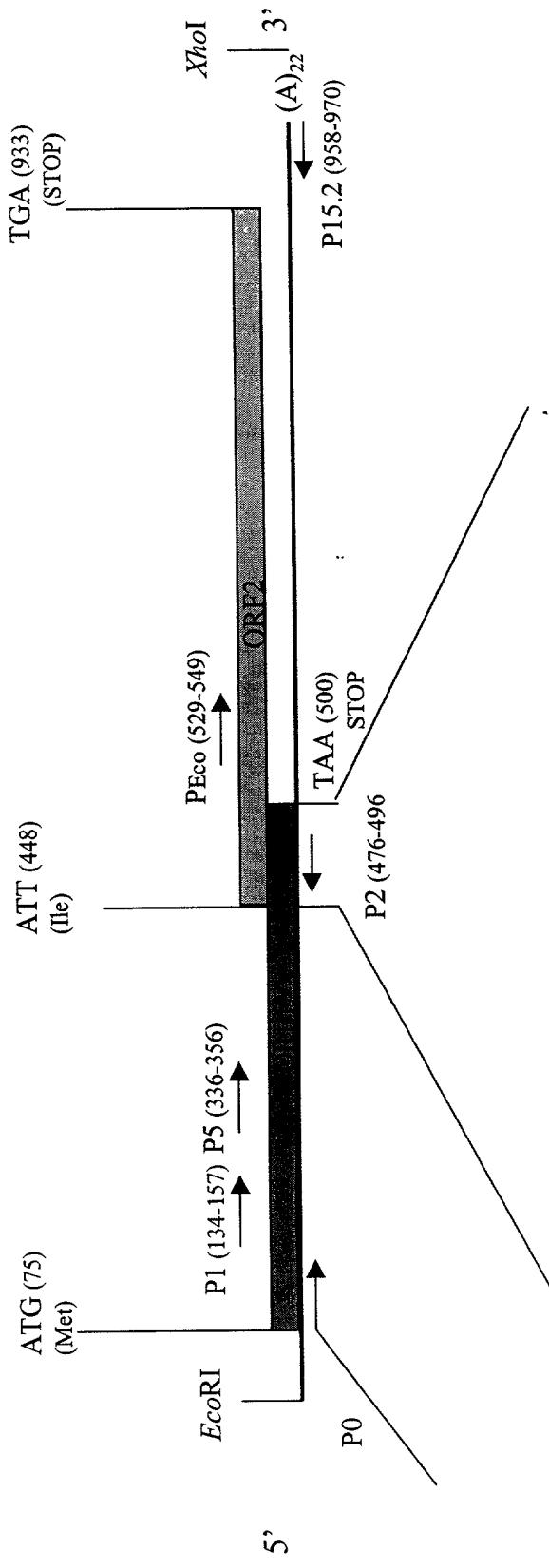
21) Diagnostic test for the detection of antibodies against *Babesia canis* associated antigenic material, characterised in that said test comprises a protein or an immunogenic fragment thereof as defined in claims 7-10.

22) Diagnostic test for the detection of *Babesia canis* associated antigenic material, characterised in that said test comprises antibodies against a protein or an immunogenic fragment thereof as defined in claims 7-10.

**Abstract**

The present invention relates to nucleic acid sequences encoding novel *Babesia canis* associated proteins and to cDNA fragments, recombinant DNA molecules and live  
5 recombinant carriers comprising these sequences. Furthermore, the invention relates to host cells comprising such nucleic acid sequences, cDNA fragments, recombinant DNA molecules and live recombinant carriers. Also, the invention relates to proteins encoded by these nucleotide sequences, to vaccines for combating *Babesia canis* infections comprising these proteins or genetic material encoding these proteins and  
10 methods for the preparation of vaccines. Another embodiment of the invention relates to these *Babesia canis* associated proteins for use in vaccines and to the use of the *Babesia canis* associated proteins in the manufacture of vaccines. Finally the invention relates to diagnostic tools for the detection of *Babesia canis* associated nucleic acid sequences, for the detection of *Babesia canis* associated antigens and for  
15 the detection of antibodies against *Babesia canis* associated antigenic material.

A



B

ORF2    I F V T L D L R A K E S I D S S K K A L R  
5' GCAGAAATATTTGGTACCTTAGATCTCTTAAGAGCCAAAGGAGTCATCTAAAGAAAGGCACCTACGTA 3'  
ORF1 A E Y F R H L R S L K S' Q G V N R L I stop

**Figure 1**

*EcoRI*  
**gaattcggcacgagccctgtatactgtgc**tttgcactaactccatcgtaataatttaatataataaaa      72  
 M   E   S   T   S   T   T   N   F   V   A   E   N   R   P   T      17  
 gg ATG GAG TCG ACA TCA ACA ACG ACC AAC TTT GTT GCC GAG AAC CGT CCC ACC      125  
 F   G   E   T   F   D   V   M   R   E   A   L   L   R   V   K   S   S      35  
 TTT GGT GAG ACG TTT GAT GTG ATG AGG GAA GCT TTG CTT CGT GTA AAG TCC TCT      179  
 E   R   L   A   M   L   R   A   L   A   G   M   C   G   H   R   V   L      53  
 GAA CGC TTG GCA ATG CTC AGA GCG CTT GCA GGA ATG TGC GGT CAC CGC GTC CTT      233  
 P   G   T   G   A   S   A   I   A   A   T   V   T   P   K   G   A   S      71  
 CCT GGC ACT GGT GCT TCT GCG ATA GCG GCA ACG GTA ACC CCA AAG GGG GCT TCG      287  
 M   K   L   K   P   P   R   P   Q   S   T   K   S   P   E   L   R   E      89  
 ATG AAG CTT AAA CCA CCG CGT CCG CAG TCA ACG AAG TCT CCG GAG CTC AGG GAG      341  
 L   S   R   K   I   R   E   M   N   K   T   I   S   Q   E   S   A   R      107  
 CTG TCA CGG AAG ATT CGC GAA ATG AAT AAG ACT ATA AGT CAG GAA TCA GCT CGG      395  
 V   N   H   R   L   P   E   G   H   P   L   L   E   K   R   A   E   Y      125  
 GTA AAC CAC CGG TTG CCG GAA GGC CAC CCT CTC TTA GAG AAG CGG GCA GAA **TAT**      449  
 F   R   H   L   R   S   L   K   S   Q   G   V   N   R   L   I   \*      141  
 TTT CGT CAC CTT AGA TCT CTT AAG AGC CAA GGA GTC AAT AGA CTC ATC TAA G      501  
 aaggcaactacgttaggtaccgtgcctctatgaggaatacgaaccgactagtgcacaatagacgaccgaggttcta      573  
 ccaaaggtagagccgtactctaattaccattcgcccgacggatcgcatgacaacgtgaaatctttaga      645  
 ccacgcggacgggttatccgtcaaattgtactttggcagttacggaaactcctgatctcgatttatagtca      717  
 aacttctacaccttgaagggtggcgaggaaggagatgtacgtgctcaacaccataaggagcaagcttg      789  
 ctactctatccgttacctccagctatacgtgcactgcactcaggtaaggctgtattcgttagaatac      861  
 tgcaaaaccaggatatcgctcgaggcacgcctcaccggactacgtccgagggtgaccctaacgggctgctga      933  
 actaggttcagccagcgttccgtgagttatgtcattccgggtcctcggggccggccagttcgactgg      1005  
 ttaggtttgcctactagagtacttgcacgcgcgaagcgcctccgttcaaagaacgcgcgaagccctagca      1077  
 gaaaaatgcgagggcatgactttcgagtcaaaaaaaaaaaaaaaaactcgag      1135  
XhoI

**Figure 2**

EcoRI

gaattcggcacgagccctgtatactgtgcttgcaactaactccatcgtaataattaaataataaaa72

M E S T S T T T N F V A E N R P T 17  
gg ATG GAG TCG ACA TCA ACA ACG ACC AAC TTT GTT GCC GAG AAC CGT CCC ACC 125

F G E T F D V M R E A L L R V K S S 35  
TTT GGT GAG ACG TTT GAT GTG ATG AGG GAA GCT TTG CTT CGT GTA AAG TCC TCT 179

E R L A M L R A L A G M C G H R V L 53  
GAA CGC TTG GCA ATG CTC AGA GCG CTT GCA GGA ATG TGC GGT CAC CGC GTC CTT 233

P G T G A S A I A A T V T P K G A S 71  
CCT GGC ACT GGT GCT TCT GCG ATA GCG GCA ACN GTA ACC CCA AAG GGG GCT TCG 287

M K L K P P R P Q S T K S P E L R E 89  
ATG AAG CTT AAA CCA CCG CGT CCG CAG TCA ACN AAG TCT CCG GAG CTC AGG GAG 341

L S R K I R E M N K T I S Q E S A R 107  
CTG TCA CGG AAG ATT CGC GAA ATG AAT AAG ACT ATA AGT CAG GAA TCA GCT CGG 395

V N H R L P E G H P L L E K R A E Y 125  
GTA AAC CAC CGG TTG CCG GAA GGC CAC CCT CTC TTA GAG AAG CGG GCA GAA TAT 449

F V T L D L L R A K E S I D S S K K 143  
(T) TTC GTC ACC TTA GAT CTC TTA AGA GCC AAG GAG TCA ATA GAC TCA TCT AAG AAG 504

A L R R Y R A S M R N T N R L V H N 161  
GCA CTA CGT AGG TAC CGT GCC TCT ATG AGG AAT ACN AAC CGA CTA GTG CAC AAT 558

R R P V L P K V E P D S N L P F G Q 179  
AGA CGA CCA GTT CTA CCA AAG GTA GAG CCT GAC TCT AAT CTA CCA TTC GGC CAG 612

R R S R M T T W N L R P R R T G Y P 197  
CGA CGG AGT CGC ATG ACA ACN TGG AAT CTT AGA CCA CGC CGG ACN GGT TAT CCG 666

S N G T L A V T E L L I S I Y R S N 215  
TCA AAT GGT ACT TTG GCA GTT ACG GAA CTC CTG ATC TCG ATT TAT AGA TCA AAC 720

F Y T L K V V E E G R C T C C N T H 233  
TTC TAC ACC TTG AAG GTG GTC GAG GAA GGG AGA TGT ACN TGC TGC AAC ACC CAT 774

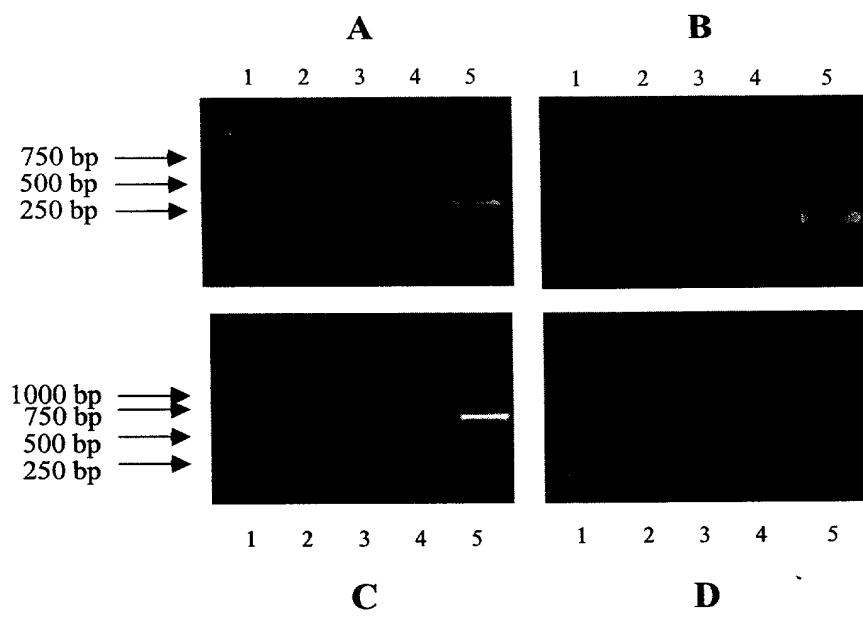
K E Q A L L L S G Y L Q L Y R A L 251  
AAG GAG CAA GCT TTG CTA CTC CTA TCC GGT TAC CTC CAG CTA TAT CGT GCA CTG 828

H S V G R S V F V E Y C K T R I C V 269  
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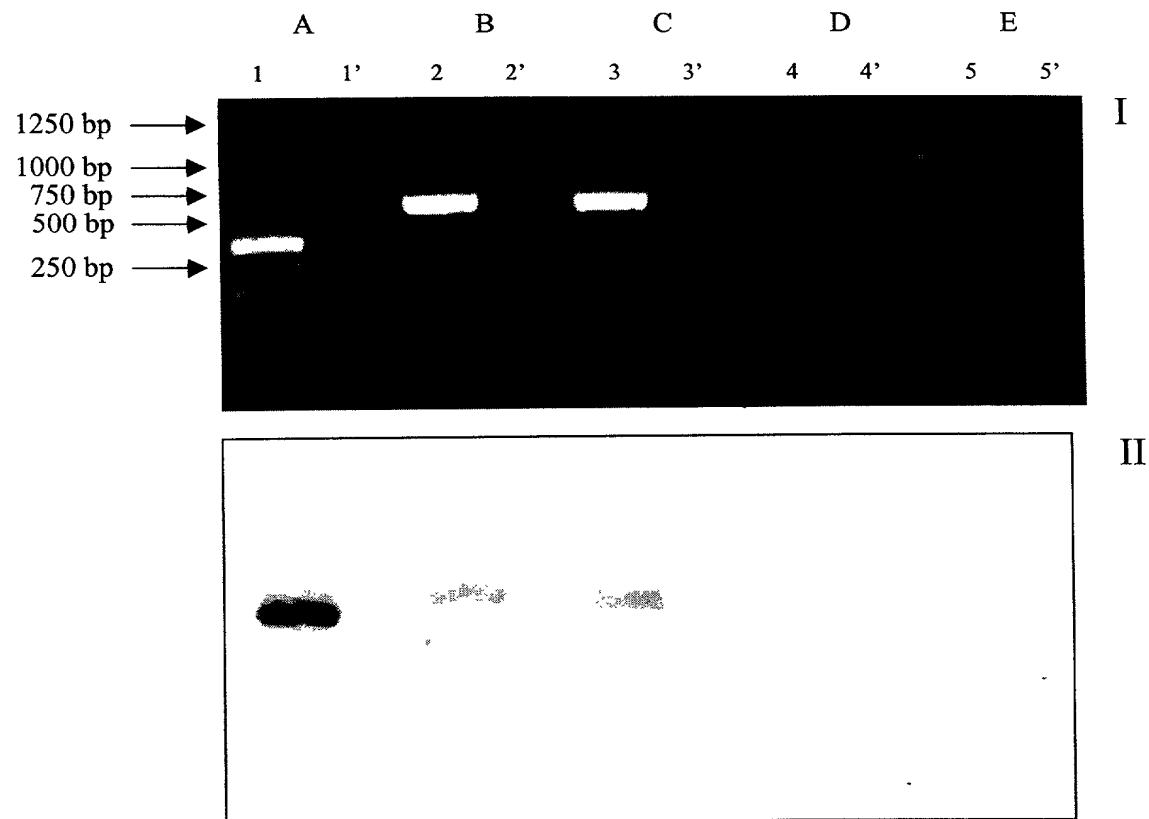
E A R L T G L R P R V T L T G C \* 285  
GAG GCA CGC CTC ACC GGA CTA CGT CCG AGG GTG ACC CTA ACN GGC TGC TGA A 934  
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gtagggttgcctactagacttgcacgcccgaagcgccctccgttcaaagaacgcgcagccctagcag 1078  
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XbaI

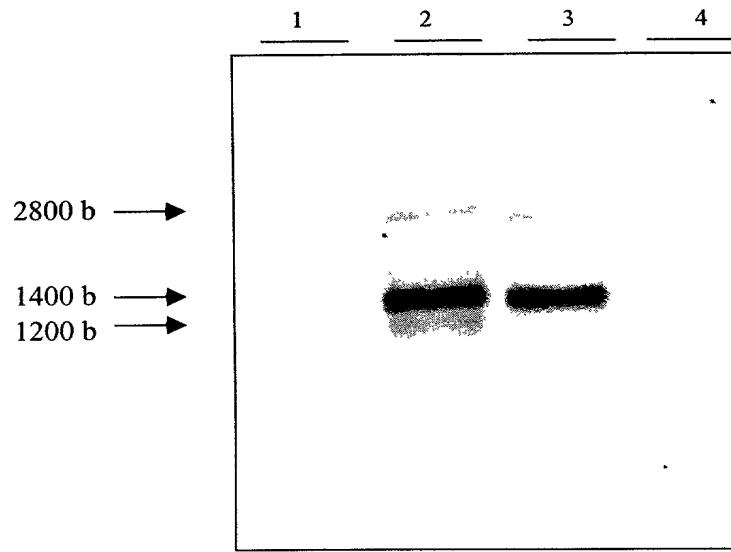
Figure 3



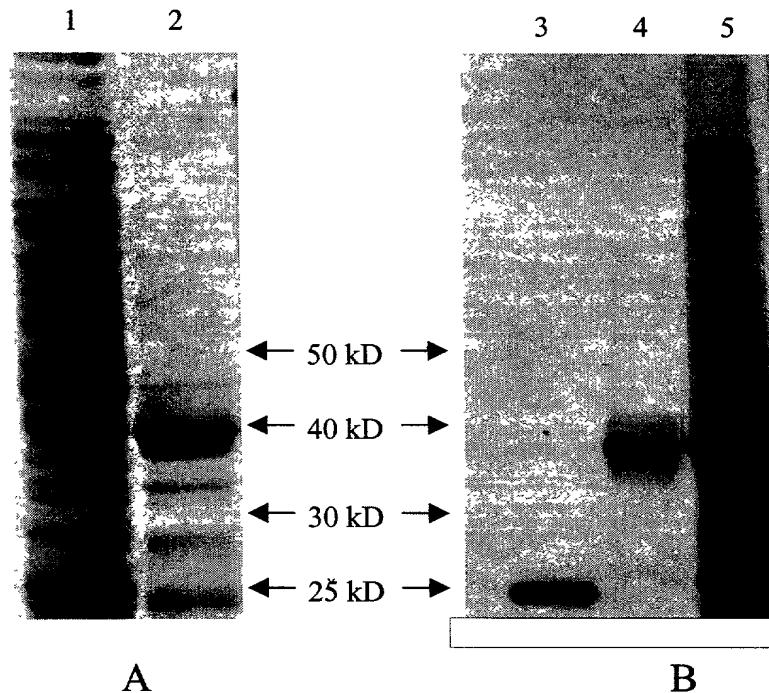
**Figure 4**



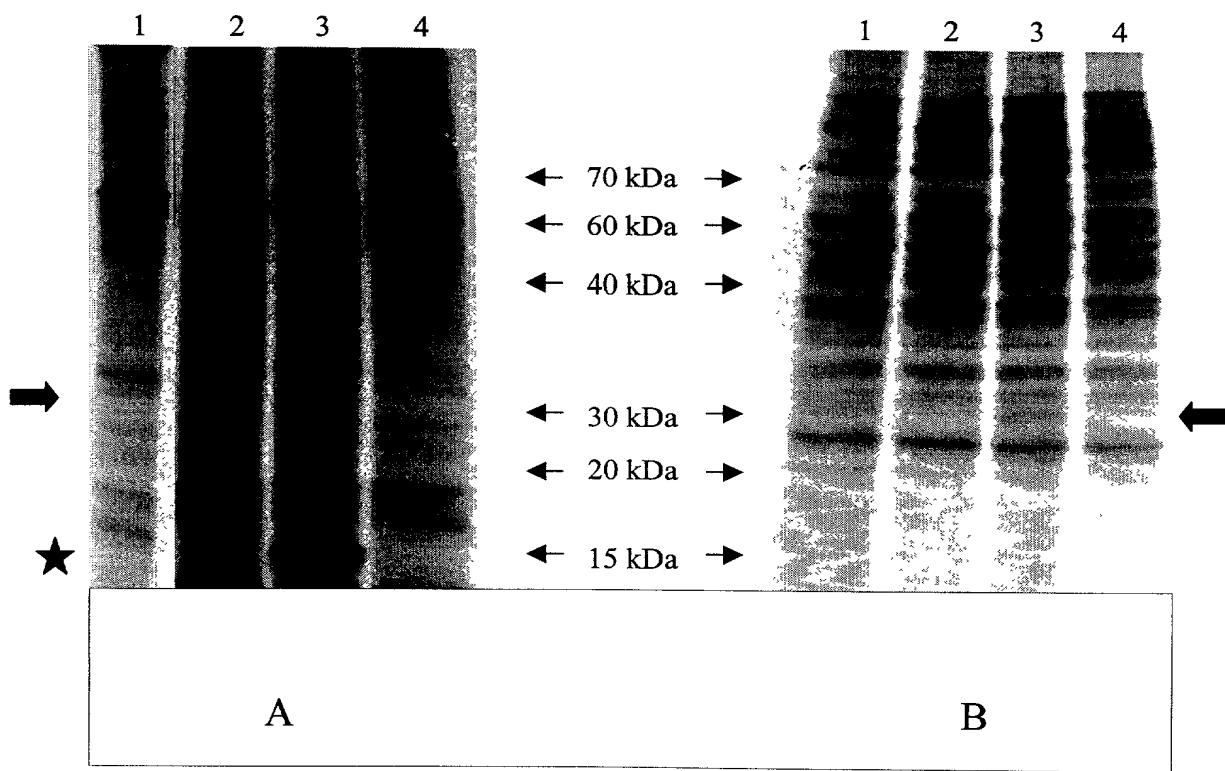
**Figure 5**



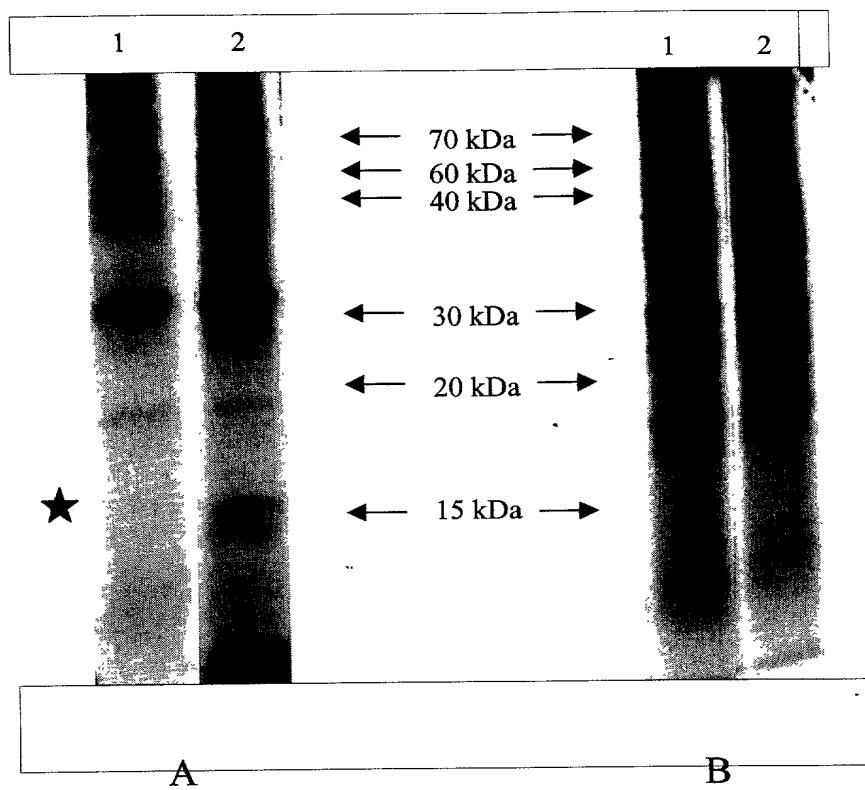
**Figure 6**



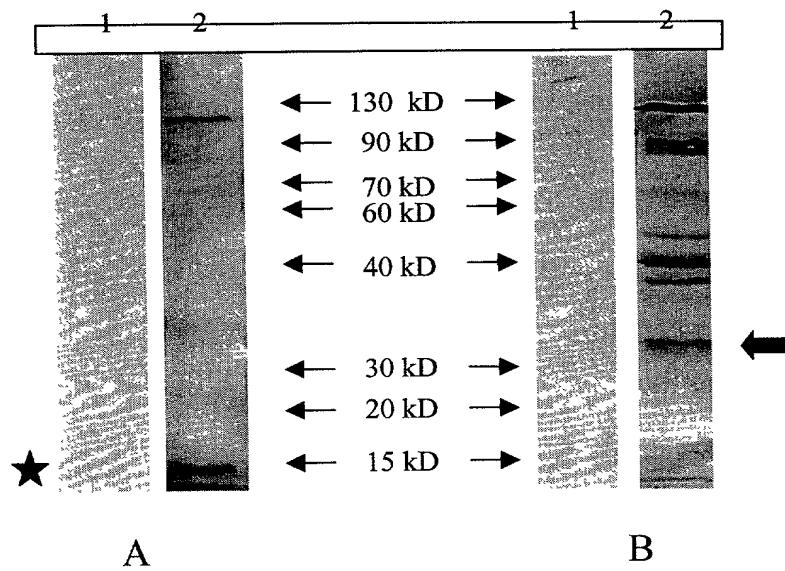
**Figure 7**



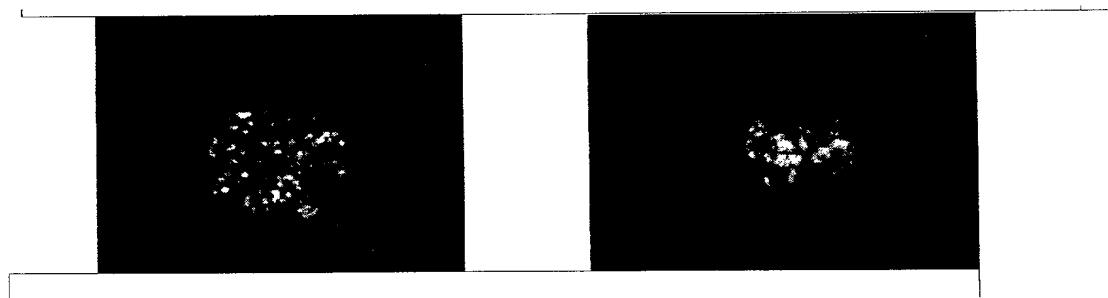
**Figure 8**



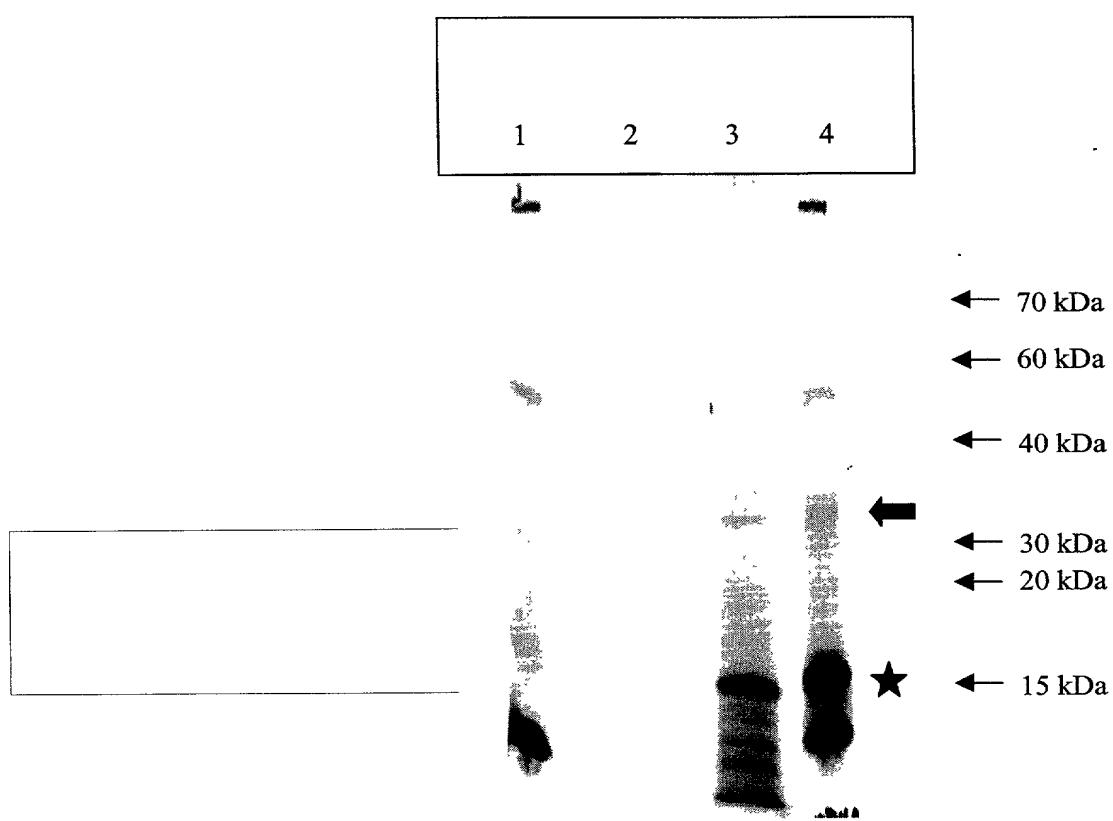
**Figure 9.**



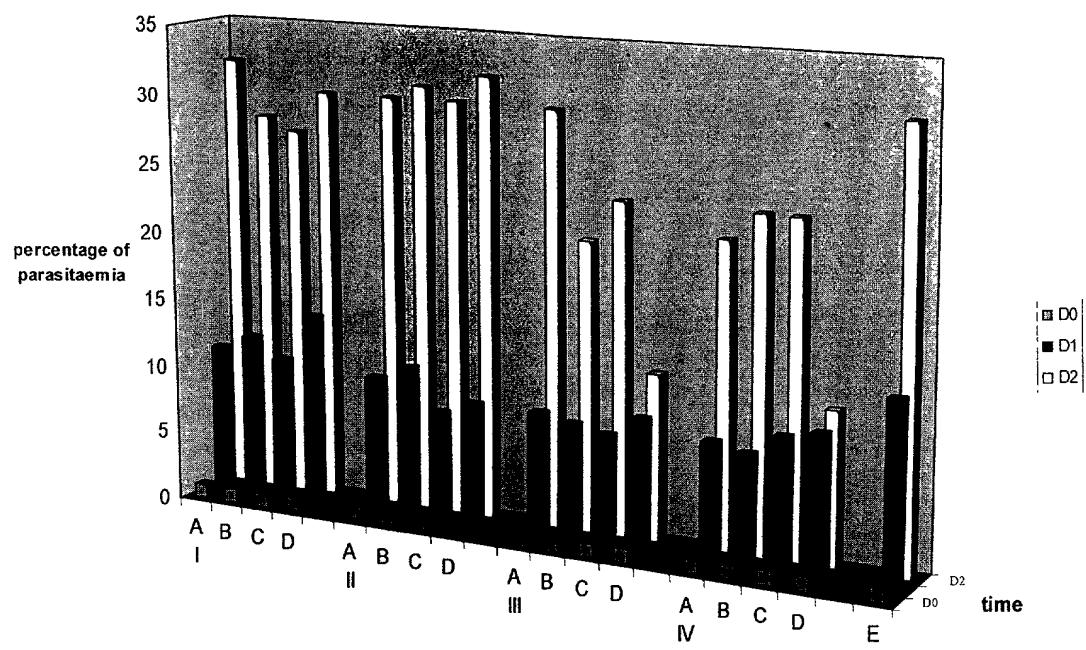
**Figure 10**



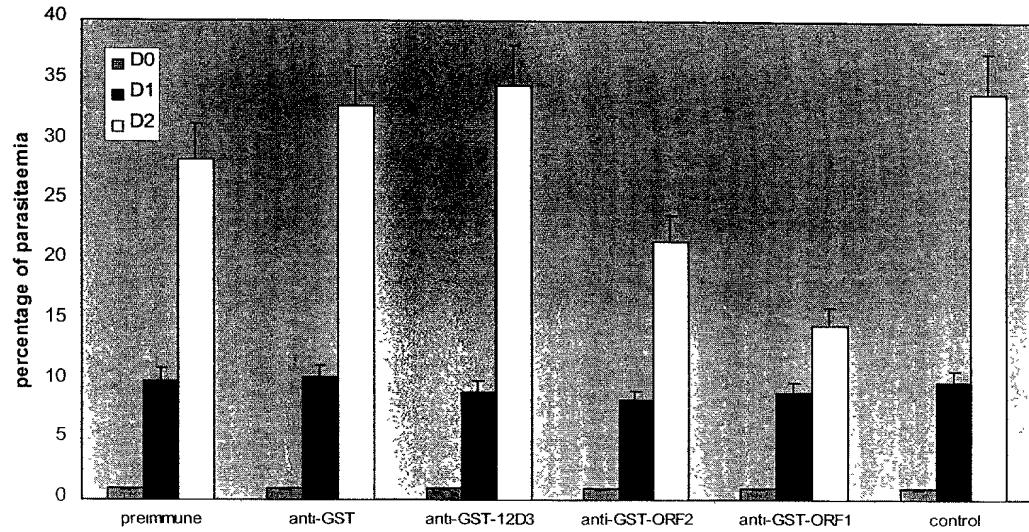
**Figure 11**



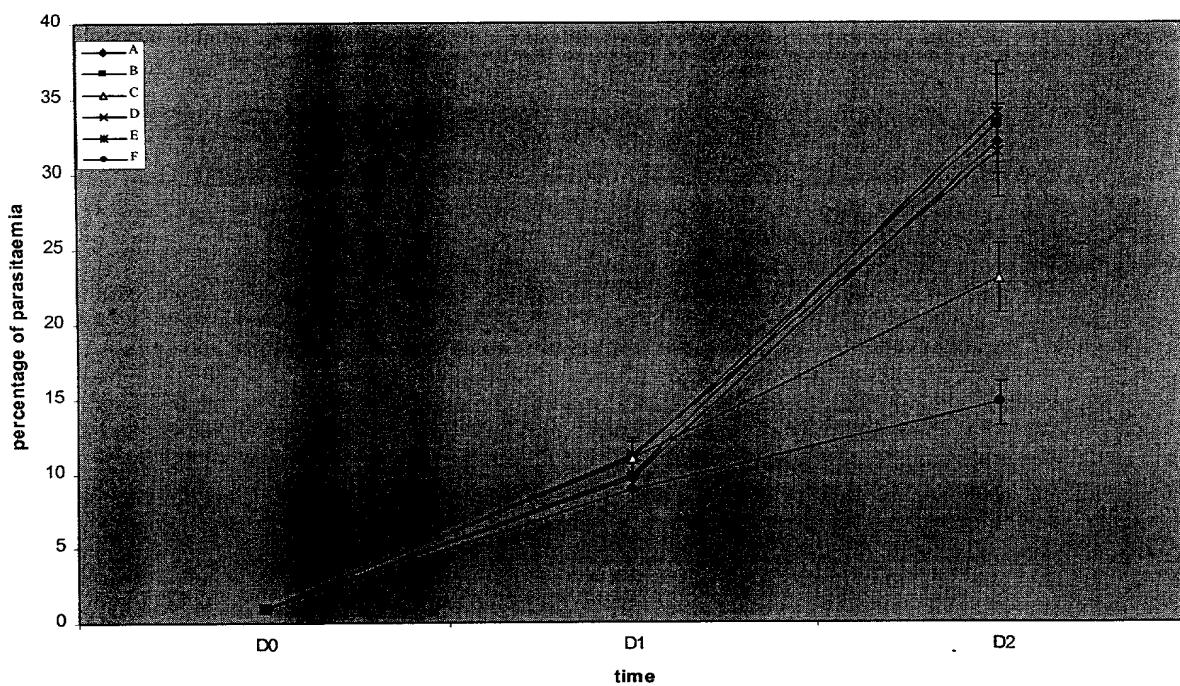
**Figure 12**



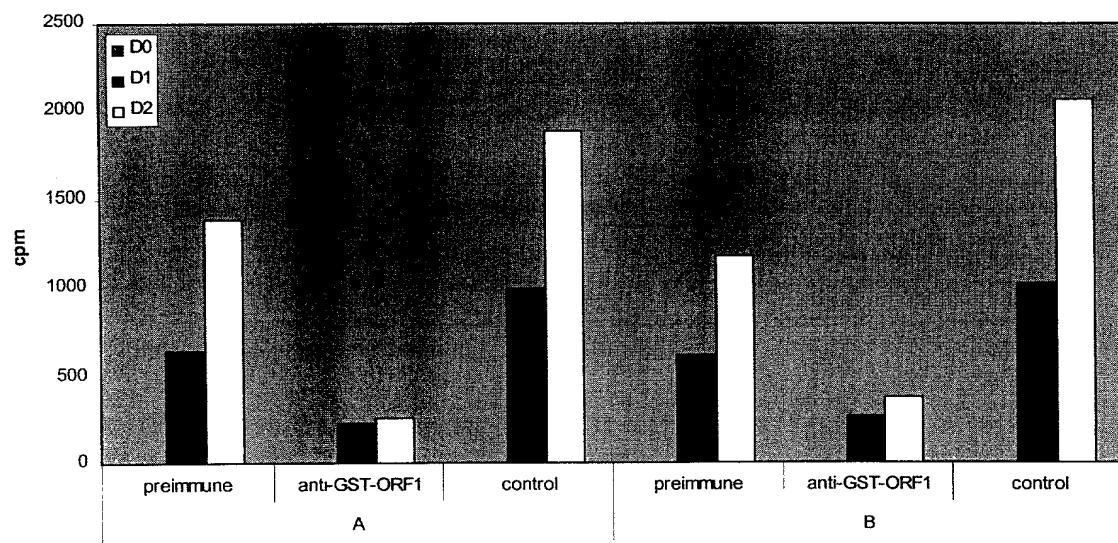
**Figure 13**



**Figure 14**

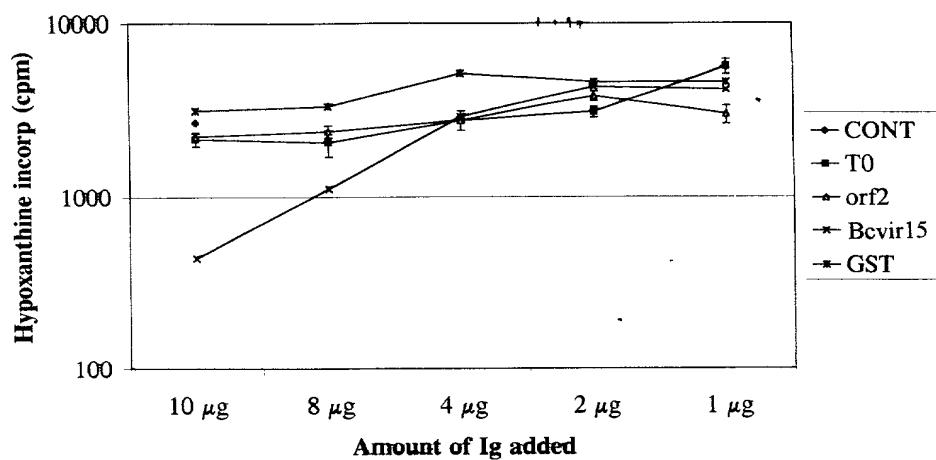


**Figure 16**

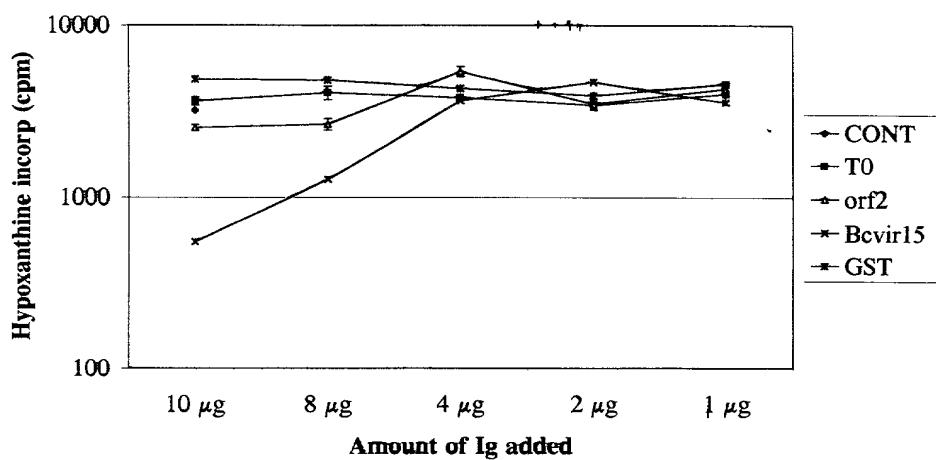


**Figure 15**

### Inhibition *in vitro* *B. canis* A



### Inhibition *in vitro* *B. canis* B



### Inhibition *in vitro* *B. rossi* M

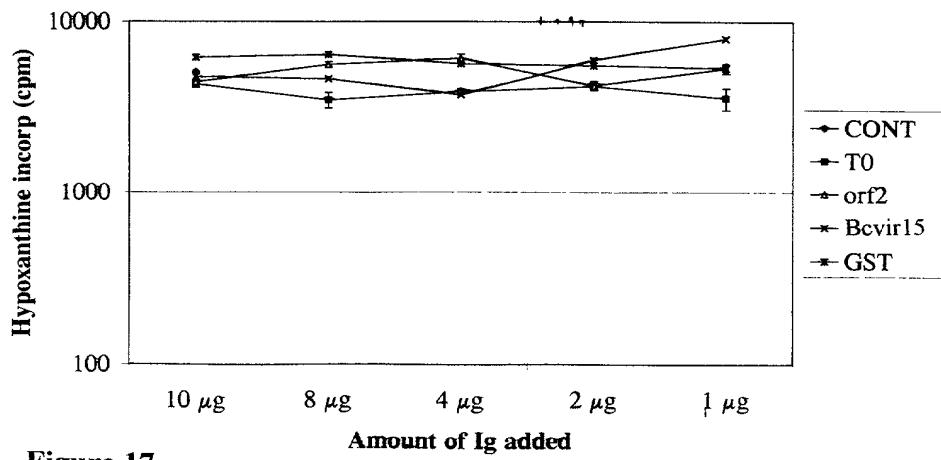


Figure 17